# Antibiotic resistance in humans and pigs : is there a relation ?

# Antibiotic Resistance In Humans And Pigs

## Is There A Relation ?

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# ANTIBIOTIC RESISTANCE IN HUMANS AND PIGS

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# IS THERE A RELATION ?

PROEFSCHRIFT

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# INTRODUCTION

## General Background

Since the clinical introduction in 1935 of the sulphonamides, followed by penicillin in the early forties, antimicrobial agents have been used extensively in human and veterinary medicine. Shortly after the introduction of antimicrobial agents, however, treatment failures were observed caused by bacteria that had become resistant to the agents used and it was realized that the emergence of resistance was an inevitable consequence of the use of antimicrobial agents. The discovery that emergence and spread of resistance was not only caused by spontaneous mutations of bacterial chromosomal DNA followed by the dissemination of a single resistant clone or strain, but that extrachromosomal bacterial genes coding for resistance could be transferred between bacteria greatly caused concern. The more so as this transfer not only readily occurs among bacteria of the same species, but also between different species. Especially the dense endogenous bowel flora of man and animals appears to be an ecological system that favours the inter- and intra-species transfer of resistance. The use of antibiotics in livestock inevitably selects for resistance in the endogenous flora of animals. This and the real possibility of transfer of either resistant bacterial strains or their genetic material to the human endogenous flora or human pathogens raised questions over whether and to what extent the use of antibiotics in food animals contributed to the occurrence of resistance in (potential) human pathogens. The increasing awareness of the importance of antimicrobial resistance and its potential public health hazard prompted the still ongoing discussion between the veterinary and medical profession about the risk of antibiotic use in animals and its possible threat to public health. In 1968 a joint committee was set up in the U.K. to examine the problem of the use of antibiotics in animal husbandry and veterinary medicine. The most important recommendation made in the Swann Report (8) was to prohibit the use of those antibiotics as growth promoters in animal feeds which are approved for therapeutic use in human and veterinary medicine. This recommendation was put into effect in 1970 in the U.K., other member states of the European Union at that time soon followed. The effect of the Swann report on the use of antibiotics in animals was, however, low. Although clinically used antibiotics were no longer used as growth promotors and were replaced by other compounds, such as virginiamycin and avoparcin, total veterinary antibiotic usage actually increased due to an increased therapeutic usage. The most important contribution

of the Swann report was that it made the veterinary profession aware of its responsibility to preserve the therapeutic usefulness of antimicrobial agents for the treatment of bacterial infections in animals and in man.

In 1989 a Dutch expert working party set up by the Institute of Public Health and Environmental Hygiene (RIVM), recommended to prohibit the registration of the newer $\beta$-lactam antibiotics, the new aminoglycosides and all fluoroquinolones for veterinary use (1). The at that time published Dutch Veterinary Drugs Act made this feasible by providing the possibility to refuse the registration of drugs for use in animals in the interest of public health, despite the fact that they might fulfil the required criteria for registration. The argument in the case of antibiotics was that the veterinary use of these agents might lead to resistance in micro-organisms (potentially) pathogenic for man. This report provoked, as could have been expected, strong reactions from the veterinary profession.

In 1990 a workshop was organised in The Netherlands to discuss the recommendations of the RIVM report of 1989 (6). Although the assumption that a decrease in the usage of antibiotics in veterinary practice should reduce the risks of transfer of resistant bacteria from animals to humans was generally accepted it remained unclear at the conference whether or to what extent veterinary use of antimicrobial agents contributed to the antibiotic resistance problems in human medicine. As a result of this conference, the Dutch Royal Society for Veterinary Medicine decided to develop a veterinary antibiotic policy with one national veterinary antibiotic formulary, this in order to safeguard the efficacy of veterinary antibiotic therapy for now and in the future and to take responsibility for public health (2). This policy was published in 1994 (3) and the first concept of a formulary is now circulating among the profession.

In December 1991 at the Fedesa Symposium in London, organised as a follow-up of the RIVM workshop, both medical and veterinary experts on antibiotic resistance discussed the relation between the usage of antimicrobials in veterinary medicine and the resistance encountered in human pathogens. The opinions differed widely. The veterinary microbiologist Espinasse was the only one who advocated that the usage of specific antimicrobial agents should be restricted to human medicine only (4). Considering the available evidence others, including the medical microbiologists, came to the conclusion that animals as a reservoir of antibiotic resistance were of negligible importance for man (5,7,9).

The discussions mentioned illustrate the ongoing and increasing concern about the veterinary antibiotic usage as a potential human public health risk factor, i.e. that selection and transfer of resistant bacteria or resistance genes from animals to man may

ultimately result in a loss of therapeutic efficacy of antibiotics in human medicine.

In this thesis several aspects of the relationship between antimicrobial resistance in the intestinal flora of man and pigs, living in the same geographical area, were explored. Three groups of faecal samples of pigs were investigated. The first group, described in chapter II, consisted of samples collected at a single pig fattening farm. During a period of 11 months (from June 1991 to April 1992) faecal samples were collected from pigs housed in different compartments of the farm. The second group, described in chapters IV and VII, consisted of mixed faecal samples once-only collected at different farms (from July 1991 to December 1991) and the third group, described in chapter VII, consisted of floor dropping collected from trucks transporting fattening pigs (from May 1992 to July 1992).

Besides, three groups of humans with different intensity and frequency of contact with pigs or pig products were investigated, i.e. pig farmers, abattoir workers and (sub)urban residents.

The prevalence and degree of antibiotic resistance of *Enterobacteriaceae* (in particular *Escherichia coli*) isolated from the intestinal flora of pigs and humans were determined, as well as the antibiotic susceptibilities of *E. coli* isolates from the same faecal samples. The results were related to risk factors such as recent use of antibiotics and direct or indirect contact with pigs. In addition to these phenotypic markers, the resistance plasmid profiles were analyzed and transfer experiments were performed *in vitro* and *in vivo* to study the capacity of *E. coli* strains isolated from pigs and humans to transfer their resistance plasmids.

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## Outline of the thesis

In *chapter I* the literature is reviewed concerning the prevalence of antibiotic resistance in the intestinal flora of food animals. The potential public health risk of the possibility of transfer of resistance between animals and man is discussed. The problems caused by antibiotic resistance, the mechanisms involved, and the emergence, selection and dissemination of resistant bacteria and resistance genes are especially emphasized.

*Chapter II*, entitled "Monitoring antibiotic resistance of *Enterobacteriaceae* isolated from the faecal flora of fattening pigs", describes the results of a study into the prevalence and the degree of antibiotic resistance of faecal *Enterobacteriaceae* as well as the susceptibility of *E. coli* strains isolated, during an 11 month period, from three groups of fattening pigs at a single farm. The variation over time and the reproducibility of sampling were the subject of the study.

In *chapter III*, entitled "Resistance in faecal *Escherichia coli* isolated from pig farmers and abattoir workers", the prevalence and degree of antibiotic resistance of faecal *E. coli* isolated from pig farmers and abattoir workers are compared with a control group isolated from (sub)urban residents.

In *Chapter IV*, entitled "Antibiotic resistance among *Escherichia coli* isolated from faecal samples of pig farmers and pigs", the results obtained with faecal samples of pigs and of pig farmers (chapter III) at the same farm are presented. The prevalence and degree of resistance of faecal *E. coli* and the susceptibility(patterns) of *E. coli* isolates of both groups of isolates are compared.

*Chapter V*, entitled "*In vitro* transfer of antibiotic resistance between faecal *Escherichia coli* strains isolated from pig farmers and pigs", describes a study in which a selection of the strains from the previous study (chapter IV) was used. Biotypes, plasmid patterns, transferability of antibiotic resistance and isolation and typing of plasmids were used as phenotypic and genotypic markers.

In *chapter VI*, entitled "*In vivo* transfer of resistance plasmids in rat-, human- or pig-derived intestinal flora using a rat model", germ-free rats associated with different specific *Enterobacteriaceae*-free intestinal floras were used to study the *in vivo* transfer of resistance plasmids. The animals were colonized with porcine and human donor strains. The influence of the different intestinal floras on resistance transfer was investigated.

*Chapter VII*, entitled "Surveillance of antibiotic resistance among pigs using faecal floor droppings from trucks transporting fattening pigs", was performed to investigate whether floor droppings collected from trucks transporting pigs to the slaughterhouse could be

used in a resistance surveillance programme to replace the cumbersome and time consuming collection of rectal samples at different farms. The prevalence and degree of antibiotic resistance and susceptibilities of faecal *E. coli* from the truck samples were compared with the results of the faecal samples collected at farms in the same geographical area (chapter IV).

In *Chapter VIII* the results of the studies presented are reviewed and discussed. Recommendation for future research are formulated.

REVIEW OF THE LITERATURE

AIMS OF THE STUDY

# REVIEW OF THE LITERATURE

## Introduction

Next to the application of the principles of hygiene and aseptic techniques, the introduction of antibiotics and the development of vaccines has been one of the most important advances in human and veterinary medicine.

The first antimicrobial agent, which is still in use today, was discovered by chemists in Germany, testing certain kinds of dye for antimicrobial activity in pursuit of Paul Ehrlich's concept of chemotherapy (36,63,78). Ironically, further investigation of an effective molecule, the red dye prontosil, showed that it was the part of its structure without dyeing properties that was responsible for the antimicrobial effect. This was the sulphonamide portion (63,78). Once it was established that sulphonamides were effective antibacterial drugs, an enormous programme of chemical synthesis and modification was undertaken, resulting in many related compounds entering the medical armamentarium.

After this chemical approach, a microbiological one arose as a result from Sir Alexander Fleming's observation that a fungus contaminating an agar plate with staphylococci was able to inhibit the growth of these and other, mainly Gram-positive, bacteria (36,63). The realisation of the fact that one micro-organism could produce a substance which was a potent killer of other bacteria had an enormous impact on the pharmaceutical industry. Huge programmes were set up to search for other soil fungi and bacteria producing antibacterial substances (36). Soon streptomycin, chloramphenicol and the tetracyclines were discovered (78). All these molecules appeared to be effective, even though they were not always as safe as penicillin. These compounds are the true antibiotics: natural substances produced by micro-organisms which in low concentrations kill or inhibit growth of other micro-organisms and do not harm the host (63).

A breakthrough in antibiotic development was a combination of the microbial approach and the chemical route. Naturally occurring antibiotics were chemically modified by substituting different side chains or incorporating atoms to improve their antibacterial activity or their pharmocokinetic properties. This has led to the development of large numbers of antibiotics, such as penicillins and cephalosporins (36,38,116). This chemical synthesis has made the differentiation between natural substances and chemically derived drugs obsolete. Therefore, nowadays the word antibiotic is used in the broad sense including chemically produced antimicrobial agents, such as sulphonamides and quinolo-

nes.

Antibiotics have proved to be very effective and safe for treatment of bacterial infections in man and animals. Unfortunately, the use of antibiotics in both human and veterinary medicine exerts a strong selective pressure inducing resistance to the antibiotics used or to related antibiotics among bacteria. Consequently medical and veterinary clinicians were forced to change their choice of antibiotics at relatively frequent intervals. This was until recently not considered a major problem as new compounds were available to replace the "lost" antimicrobial agent. The continuous stream of new antibiotics coming on the market during the last decades might have given the impression, that there would always be new antibiotics available to kill bacteria resistant to the older ones and the increasing resistance problem was ignored. However, after a half-century of virtually complete control over microbial diseases in the developed countries, the mid eighties have brought a worldwide resurgence of bacterial diseases (15).

An important factor in this phenomenon is the acquisition of antibiotic resistance genes by bacterial pathogens. Recently, bacterial strains resistant to most available antibiotics were identified among clinical isolates of enterococci (29,82). This increase of resistance against several non-related antibiotics (multi resistance) among pathogenic bacteria in hospitals and in veterinary isolates, as well as the slowing-down in the introduction of totally novel antibiotics, has lead to a reappraisal of the significance of antibiotic resistance.

Nowadays antibiotic resistance is a problem in veterinary medicine and a major problem in hospitals, because infections caused by resistant bacteria result in prolonged morbidity and mortality from treatment failures and increased costs as newer, more expensive and often more toxic antibiotics are needed to treat common infections. As resistance spreads, involving more antibiotics and more pathogens, infections, which cannot be treated effectively with antibiotics, may become more prevalent (52,61,80). Another reason for concern is that new antimicrobial agents will be used against multi resistant pathogens (15,17,81). This practise will virtually ensure, that eventually resistance against the new antibiotics will appear in the currently multi resistant bacterial species.

Although the emergence of resistance may be an inevitable consequence of the use of antibiotics, there is clear evidence that it need not to occur to the degree that it does at this moment. The emergence of resistance and the selection and dissemination of resistant bacteria or their resistance genes is greatly enhanced by the amount of antibiotics used, as this provides resistant micro-organisms with a powerful neo-Darwinian advantage. As the use of antibiotics and the transmission of micro-organisms are factors over which we have control, intervention is feasible (15,18,49,56).

The different aspects of the consequences of antibiotic usage and resistance in the microflora of animals and the possible public health risks involved will be reviewed.

## Antibiotic resistance

Antibiotic resistance is the ability of micro-organisms to survive or even to grow in a concentration of an antibiotic that would inhibit or kill susceptible bacteria (63,103). When an antibiotic is present in the bacterial environment, resistant bacteria will soon be the predominant organisms in that population. In considering the problem of antibiotic resistance of bacteria, it is the emergence of resistance in bacteria belonging to a susceptible species that is of concern and not unsusceptibility.

*Unsusceptibility*

Unsusceptibility, also called intrinsic or natural resistance, implies that all bacteria belonging to a certain species are not affected by a certain antibiotic. This can be caused because the target sites of that compound cannot be reached or are absent in that species (9,101,103).

*Resistance*

Resistance, sometimes called acquired resistance, has indeed to be acquired. This means that bacteria belonging to a species normally susceptible to a specific antibiotic are not inhibited or killed by that particular drug in the concentration usually effective (9,103). In case of resistance the organism has obtained the means to survive exposure to that particular antibiotic. Several mechanisms can be involved. The bacteria may excrete enzymes that can destroy one or several related antibiotics (30,116). Bacterial structures such as the cell wall or metabolic pathways might have been changed, resulting in the impossibility of the antibiotic to reach or bind to its target site (102,115,117).

There are three ways for resistance to emerge in a bacterial species: selection, mutation and acquisition of new genes.

*Selection of resistance*

In case of selection a minority of the population requires a higher dose of a certain antibiotic to be inhibited or killed or was already resistant before the introduction of that particular agent (21,36,46,63). During exposure to that agent the resistant or less suscep-

tible bacterial strains have a selective advantage and as a result these strains become predominant in the bacterial population. The classical example of selection is the sudden emergence of resistance in *Staphylococcus aureus* to penicillin shortly after its introduction (12,63). Also the susceptibility of gonococci to penicillin has gradually decreased since this agent was used for treatment of this disease and consequently the required effective doses for treatment had to be increased (36). One of the causes was the selection of strains with a diminished permeability of the cell wall for penicillin (116). The increase of resistant clinical human *Campylobacter* spp. is also most likely a result of selection caused by exposure to enrofloxacin, used to treat *E. coli* infections in poultry (32,33). *Campylobacter jejuni* and *Campylobacter coli* are common intestinal inhabitants of poultry and people can be infected after consumption of insufficiently cooked or unhygienic treated poultry meat (33,39). However, treatment of human *Campylobacter* infections with quinolones has contributed to the problem of resistant strains (2,87).

*Mutation*

Mutations are changes in the DNA sequence of the bacterial chromosome, that usually occur during replication (27,101,103). If the mutation(s) cause the bacterium to become resistant, under antibiotic pressure these mutated bacteria can be selected and become the predominant population. This resistance is only passed on vertically to their offspring: clonal dissemination of resistance. Mutationally altered bacteria are often metabolically deranged (sick) and are at a selective growth disadvantage compared to the wild types under normal growth conditions. Hence, in the absence of a selective advantage, such as the usage of antibiotic(s), bacteria with chromosomal mutations mostly disappear spontaneously from the population (103). However, when the selective pressure is maintained (e.g. antibiotics are used) such mutants may establish themselves in the population and become a threat to human and animal health. So is the diminished susceptibility of gonococci also caused by a mutation reducing the affinity of the penicillin-binding proteins in the gonococcal cell membrane (108). Alarming is the recent observation that a single point mutation in $\beta$-lactamase genes, like TEM-1, extends the activity spectrum of the produced $\beta$-lactamases from a few broadspectrum penicillins and first generation cephalosporins to nearly all the $\beta$-lactam antibiotics available at this moment for clinical use (8).

*Acquisition of new genes*

The most important way by which bacteria may develop antibiotic resistance is by acquisition of new genes by uptake of foreign genetic material (3,101,103). It seems easier for

a micro-organism to pick up one or several existing resistance genes, than for a new resistance gene to emerge, which requires many mutations. Gonococci became resistant to penicillin after acquiring the TEM ß-lactamase gen from *E. coli* (108). One might wonder where and why these genes conferring resistance to antibiotics originated from. One should bear in mind that antibiotics are naturally occurring substances produced by soil microbes (63), comparable to the production of benzylpenicillin by the fungus *Penicillium notatum* or chloramphenicol by the bacterium *Actinomyces venezuelae*. These compounds are toxic, even to the micro-organisms that produce them, so in order to protect themselves antibiotic-producing bacteria also have resistance determinants. It has been speculated that resistance genes in other bacteria have been derived from antibiotic producing soil microbes by horizontal gene transfer or by spontaneous mutation of bacterial genes, and that this mutation was maintained under selective pressure of exposure to antibiotics produced by soil bacteria (4,63). Antibiotic resistance has been observed in bacterial pathogens from the pre-antibiotic era (36). Siddall (98) found resistance to cefoxitin and clindamycin in 140-year old *Clostridium* strains. Other studies have described the presence of resistance plasmids in *Enterobacteriaceae* isolated from human infections before medical use of antibiotics (21,46).

*Plasmids*

Although most of the bacterial genes are found on the bacterial chromosome, some genes can be found on relatively small, circular, self-replicating DNA molecules, which are independent of the chromosome and are called plasmids (83,103). A single bacterium can carry several types of plasmids and those that harbour the genes for antibiotic resistance are called resistance factors (R-factors) or resistance plasmids (R-plasmids). Plasmids tend to establish themselves stably in bacteria, even in the absence of a selective pressure, because genes essential for survival of the organism are also often located on plasmids. Plasmids can multiply independently of the bacterium that harbours them and, more important, they can be transferred to other bacteria, of the same and of other micro-organisms, by several ways: transformation, transduction, or conjugation. This allows bacteria to adapt themselves quickly to changes in their environment, which means that if antibiotics are polluting their ecological niches bacteria become resistant. Usage of such an antibiotic on a large scale may than cause a rapid, maybe even worldwide, dissemination of resistance (5,107). Plasmid mediated resistance has been observed for nearly all antibiotics: aminoglycosides, cephalosporins, chloramphenicol, penicillins, sulphonamides, tetracyclines and trimethoprim (13,61). Exceptions to date are the fluoroquinolones,

polymyxins, nitroimidazoles and nitrofurans (13,78,86).

*Conjugation*

Conjugation is the bacterial version of a sexual encounter. Two bacteria come into close contact and exchange plasmids via a bridge or mating pore from a donor to a recipient strain (4,76,83). Conjugation seems to be the most important way of transfer of resistance. However, only genes on specialized conjugative plasmids can be exchanged by conjugation. Bacteria have solved this problem by another molecular tool: transposons.

*Transposons*

Transposons are small mobile DNA fragments that can jump between plasmids or even from chromosomes to plasmids and vice versa (4,103). Unlike plasmids, transposons do not rely on a particular host cell or any specific host DNA in order to exist or multiply. This additional step allows virtually all resistance genes to be exchanged by conjugation, making for a very efficient way of transferring resistance and easy for R-plasmids to pick up new resistance genes passing through bacteria. Recently, transposons have been discovered which cannot only transfer themselves from one bacterium to another, but can also mobilize co-resident plasmids and make non-transferable plasmids transferable: conjugative transposons. Conjugative transposons are widespread among clinical isolates and the transfer of some is even regulated by tetracycline (93).

*Multi resistance*

If several resistance genes are located on a single plasmid then the bacterium becomes multi resistant: resistant to two or more unrelated antibiotics. The use of one antibiotic always favours the spread of plasmids containing resistance genes for that particular antibiotic, but in case of multi resistance the genes conferring resistance to several other antibiotics are simultaneously transferred. Consequently, all bacteria harbouring the new plasmid also show resistance to the antibiotics encoded by that plasmid. This leads not only to decreased usefulness of the selecting agent, but also to that of the other antibiotics (9). Thus, changing the use of one antibiotic does not stop the selective pressure unless all relevant drugs are withdrawn.

*Transduction*

Transduction is a process in which a viral vector, a bacteriophage, is used to shuttle resistance genes from a resistant bacterium to a susceptible one (36).

*Transformation*

In case of transformation, pieces of naked DNA containing resistance genes are released from dead bacteria and taken up by other competent bacteria (36). As a number of antibiotic preparations have been shown to be contaminated with chromosomal DNA of the antibiotic-producing micro-organism, this might be a more important source of resistance than expected up to now (111).

## Dissemination of resistance

Despite the fact that resistance always has been present in the bacterial population, before the first penicillins came into clinical use, selection and spread of resistance has increased enormously since during the last 50 years a large number of antibiotics with distinct mechanisms of action have come available. The usage of enormous quantities of these drugs, not only for therapy and prevention of bacterial diseases in man and animals, but also as growth promoting agents in animal husbandry and for crop protection in agriculture, has challenged the entire prokaryotic world. As a result resistant bacteria can be found anywhere in the environment of man and animals: in water and soil, on pastures, in animal waste and on meat(products), fruits and vegetables (68,75,88,106). Hence, transferable resistance is not confined to pathogenic micro-organisms. As a matter of fact the problems of resistance encountered in pathogenic bacteria could be considered as the top of the resistance iceberg (24,40,62).

The acquisition of resistance by a bacterium and the spread of a resistant strain should be considered as two quite separate phenomenons. The most dramatic problem is a major outbreak, even epidemic, caused by a single strain which has acquired antibiotic resistance and probably additional factors or has encountered circumstances enabling the strain to spread widely in a particular population: hospital patients, the community at large and/or in animal populations. This clonal dissemination includes the events with *Salmonella typhimurium* type 29 and type 204 in which strains carrying plasmids conferring resistance to chloramphenicol, amongst other antibiotics, were spread among thousands of calves all over the U.K. and also affected several humans, whereas most other *Salmonella* spp. isolated at that time were sensitive to most antibiotics as at the present time (7,22, 104). Most outbreaks of human salmonellosis reported in the literature are single strain epidemics (43,73,91). The interest in salmonella infections and resistance in salmonellae is high because they are easily detectable and traceable. The final goal, however, ought to

be to make animals intended for food production free from zoonotic pathogens, such as *Salmonellae* spp., and not to keep the causal bacteria susceptible to antibiotics.

Apart from selective pressure by antibiotics, transfer of resistance genes between bacteria is especially likely when the species live in close proximity. Such a situation is present in the intestinal tract of man and animals, which is inhabited by hundreds of bacterial species in high concentrations and among these there are often several (potential) pathogens. Most research on transfer of resistance among the endogenous flora of man and animals has therefore focused on the gut. Faecal samples represent the contents of the terminal colon and the rectum. This flora consists for more than 99.9% of obligatory anaerobic bacteria. Only a minority belong to facultatively anaerobic bacterial species e.g. *Enterobacteriaceae*, enterococci, lactobacilli and the so called transient flora, consisting of bacteria that are ingested and only shortly pass through the intestine (10,16). The endogenous intestinal flora is also a major factor in the natural resistance to infections with bacterial pathogens. The establishment in, or colonization of, the intestinal tract by new pathogenic bacteria, resistant or not, is combatted by the stable endogenous flora, mainly the large numbers of anaerobes (41). This protection against colonization of the intestinal tract with exogenic bacteria constituted by the intestinal flora is called colonization resistance by van der Waaij (109). This form of resistance of the gastro-intestinal tract is a complete different phenomenon compared to bacterial resistance and should clearly be distinguished. Administration of antibiotics, however, can change the composition of the intestinal flora, disturbing the colonization resistance and so permitting overgrowth by resistant endogenous micro-organisms or colonization by exogenous strains. So the presence of antibiotics in the intestinal tract is not only a selective force for selection and spread of resistance among bacteria belonging to the intestinal flora, but also favours acquisition of new bacteria from the environment, which may introduce new resistance genes.

Some people are exposed to higher risks of acquiring resistant bacteria or plasmids than others. Farmers might be exposed to antibiotics by the involuntary ingestion of medicated animal feed. It has been suggested that this, besides close contact with animals might have led to higher levels of resistant bacteria in their flora. Ojeniyi (85) and Levy *et al.* (59) showed that farm workers in close contact with poultry fed antibiotic supplemented feed acquired resistant *E. coli* strains from the birds. As the proportion of human populations who have contact with farm animals is small compared with those who do not, this route is expected to be of little importance for the general human health status (65). Nevertheless, it can have local effects. The presence of drug residues in meat and meat products is also a minor risk, in this respect possibly a theoretical one. To prevent this

risk, a specific time for withholding antibiotics from animals prior to slaughter, a withdrawal period for each veterinary product used in food animals, has been legally laid down and highly sensitive analytical methods make it possible to detect very low drug levels. Regularly checking animals at slaughter and condemnation of positive carcasses or products should protect consumers from exposure to antibiotics in meat and meat products.

The majority of the commensals are found in high numbers in the lower part of the gastro-intestinal tract in both humans and animals and this is considered a major reservoir of resistant bacteria and a pool of resistance exchange. The resistant bacteria are excreted in the faeces. *E. coli* is the most common aerobically growing species found in the faecal flora of humans and animals and it constitutes a reservoir of resistance plasmids (58,60, 71,92). Several factors can influence these reservoirs and the spread of bacteria or plasmids. *In vitro* resistance can be transferred rapidly throughout a susceptible population (47,77,112). *In vivo* transfer of resistance plasmids also occurs, but proceeds slower and at lower frequencies or only under certain conditions such as under antibiotic pressure (59,75,85,113). Plasmids encoding for antibiotic resistance have been shown to be transferred from human or animal *E. coli* to the *E. coli* resident in the intestinal flora of humans. Even in the absence of selective pressure of antibiotics these resistance plasmids may persist in the intestinal flora (14). Many studies have shown that the therapeutic administration of most antibiotics to animals and humans is associated with an increased prevalence of resistant bacteria, not only of pathogenic species, but also of bacteria belonging to the commensal intestinal flora, such as the *Enterobacteriaceae* (17,35,42, 48,90). In particular, the oral use of poorly absorbable antibiotics which reach the gut in high concentrations, such as tetracyclines, leads rapidly to the selection of resistant *Enterobacteriaceae*. Tetracycline resistance is relatively common in *E. coli* isolates from pigs (1,54,55,100). As a result, during antimicrobial therapy the numbers of resistant bacteria increase, which enhances the chance of transfer of prevalent resistance genes to other pathogenic and non-pathogenic bacteria present.

In general, resistance is more common in individuals, that are using antibiotics or recently have done so. In a population the prevalence of resistance is related to the amount of antibiotics that the population is exposed to.

## Reservoirs of resistance

*Humans*

A main cause for the presence of resistant bacteria in man is the use of antibiotics prescribed for therapy in general practice or in hospitals (51,90,110,114). However, resistant bacteria can spread from person to person. Risk factors for human to human spread are: high prevalence of resistance, antibiotic use, close contacts, crowding, low sanitary levels and/or contaminated food.

Antibiotic usage in hospitals is high, because many patients are immune-compromised caused by the underlying disease or its treatment, the increased frequency of invasive medical interventions and the prolonged survival of many patients with chronic debilitating disease. These patients represent a large reservoir of resistant bacteria which can be spread to other patients, of which many have a disturbed colonization resistance, and to the general population by person to person contact and into the environment by waste products and sewage (17,74,79). However, Linton *et al.* (70) calculated that, although the sewage from hospitals contained the highest percentage of resistant coliforms, the population outside hospitals represents the greatest reservoir of resistance factors, because of a larger sewage output. Nevertheless, it has been shown that intestinal bacteria carrying resistance factors are widely spread in the environment (19,69). Patients of general practioners showed high resistance percentages after antibiotic therapy and consequently they can spread resistance to the open population (20,72,79).

Not only in hospitals, but also in other places where people are crowded together a high prevalence of resistant bacteria in their endogenous flora is found and the risk for resistance transfer is high. Studies showed that attending day-care centres by a large proportion of children in a neighbourhood had also an effect on antibiotic resistance in the open population in that neighbourhood. Reves *et al. (89)* observed that children attending day-care centres showed high trimethoprim resistance percentages compared to adults and to children not visiting day-care centres. Singh *et al. (99)* observed the presence of an unique gene in trimethoprim resistant strains isolated from children visiting the same day-care centre and it circulated between children visiting that centre. High resistance levels in the faecal flora of a crowded population can be enhanced by environmental conditions such as poor sanitation, food contamination and because of antibiotic usage (6,53,58,94). Crowding and poor hygienic conditions facilitate person to person dissemination of resistance enhancing the levels of antibiotic resistance (6).

*Animals*

Also in animals the prevalence of antibiotic resistance in their endogenous flora is influenced by the use of antibiotics. A distinction has to be made between antibiotic usage in domestic pets and in food animals. Although people have intimate contact with pets these animals represent a minor part of the animal population and are only treated with antibiotics on an individual basis. The total amount of antibiotics used for treatment of pets is very low compared to the amounts used for humans and food animals (9). Hence, the risk of resistance selection and spread to humans has been supposed to be negligible. If normal hygienic standards are maintained pet animals do not seem to be a greater risk than any other member of the family taking antibiotics. However, Davies *et al.* (23) observed similar plasmid incompatibility groups in a small group faecal coliforms isolated from humans (n=27) and domestic pets (n=52) from which it was suggested that the plasmid populations were similar.

Food animals, like pigs, are given antibiotics not only for treatment and prevention of bacterial diseases, but antimicrobial agents are also added to the feed of these animals in low non-therapeutic doses to improve growth. This practise is considered highly effective in increasing productivity. In The Netherlands the usage of feed additives or growth promotors containing antibiotics, that are registered as therapeutic drugs either for humans or animals or showing cross-resistance with therapeutically used antibiotics, is not allowed by law. Moreover, as growth promoting antibiotics have activity against Gram-positive micro-organisms and not against most Gram-negative micro-organisms, such as *Escherichiae*, its significance in terms of contributing to antibiotic resistance in either animal or human *E. coli* populations is not exactly known, but seems absent or negligible (25,26). Breeding and raising of food animals is more and more concentrated in very large farms specializing in only one animal species instead of on small-scale family farms keeping different animals. In such settings strong management and animal husbandry techniques are emphasized as a way of minimising and controlling disease. To prevent introduction of new infectious agents and outbreaks of infectious diseases, good hygiene, vaccinations and several other policies are strictly adhered to in intensive animal farming. By employing the closed herd principle it is possible to rear and fatten animals at the same farm. If new animals have to be added to the population these animals are kept in quarantine for a period of time before they are allowed to join the herd. Another method is the "all-in, all-out" policy meaning that all animals leave the farm at the same time. Only after a period of cleaning and disinfection of the stables new animals are introduced on to the premises. All these measures are meant to prevent introduction of new

pathogenic bacteria from outside the compound, but not to prevent spread of micro-organisms within a stable or farm. Conditions are such, that when introduced, infectious diseases can spread rapidly through a large number of animals in a herd or flock, sometimes with dire consequences. Because of crowding and intensive (faecal-oral) contact between animals bacteria can easily spread from one animal to another. In case of bacterial diseases antibiotics are not only used in individual animals, but at the first sign of illness among some animals the whole group is often treated rather than waiting for the disease to be manifest in the entire group. Usage of antibiotics, however, is generally minimized to keep costs down and because prolonged usage tend not to work well in such settings. Despite this, reliance on antibiotics has become structural in intensive livestock farming (9,31). Individual animals are treated either parenterally or by oral dosing, but whole groups of animals by mixing the antibiotic through the feed or drinking water of the animals, which is called mass-medication (31). In The Netherlands the amount of antibiotics used for mass medication of food animals is approximately three times the amount used for treatment of individual animals (9). Mass-medication with antibiotics has been shown to select for the presence of large numbers of resistant *E. coli* in the intestinal flora of animals (44,66,67). In chickens, receiving tetracycline-supplemented feed, the intestinal flora changed within one week from low percentages of tetracycline resistant micro-organisms to more than 60% resistant bacteria. In contrast, chickens not fed tetracycline-supplemented feed kept low numbers of resistant bacteria in their intestinal tract (60). Also Bourque *et al.* (11) observed a significant increase in resistance to neomycin, chloramphenicol and ampicillin in pigs under the influence of antibiotic supplemented feed. Smith (100) and Ojeniyi (85) have shown that food animals represent an enormous reservoir of plasmid-bearing *E. coli*. A first risk of selecting resistant bacteria in these animals is by direct antibiotic use. This is not only due to the amount of antibiotics used, but enhanced also by the strong selection forces of mass-medication and, maybe even more, by the total lack of hygienic barriers between animals to control the dissemination of resistant bacteria.

**Transfer of resistance from food animals to man**

Because humans are often in close contact with animals or consume animal products, spread of bacterial resistance between both populations has been strongly suspected, despite the fact that resistance emerges independently in animals and in humans (28, 69,105). Zoonotic bacteria causing infections in man can be transferred from animals to

man e.g. *Salmonella* spp. and *Campylobacter* spp.

As food animals represent an enormous reservoir of plasmid-bearing *E. coli*, these strains might reach the human intestinal flora by the same way as animal pathogens are transmitted to humans e.g. by direct contact with animals and by handling or eating contaminated food products of animal origin. These strains can either colonize the humans or transfer resistance to the human intestinal flora during their passage through the intestinal tract. It still is much debated to what extent these resistant bacteria do colonize the human intestinal tract or transfer resistance. Hence, the question to what extent *in vivo* transfer of resistance factors from animals to man occurs and its importance has not been answered yet.

*Animal contact*

Healthy humans who come in close contact with farm animals show higher resistance levels in their faecal flora than persons without animal contacts. Three to five months after chickens had been fed tetracycline an increase in resistant intestinal bacteria was observed in the farm personnel, but not in their neighbours (60). Levy *et al.* (59) demonstrated that poultry *E. coli* strains from chickens receiving tetracycline-medicated feed, could colonize the human intestinal tract, but this occurred only in two out of 11 persons. Some investigators have found similar antibiotic resistance patterns between animals and man in close contact. A strong association between the resistance patterns of the *E. coli* isolates of farm families and their livestock was also observed by Fein *et al.* (34). Saida *et al.* (92) found that resistance plasmids of pig *E. coli* strains could be observed in *E. coli* isolated from humans in contact with these pigs and that those people in closest contact with the pigs harboured the highest frequency of resistance plasmids. This finding suggested the presence of a common pool of resistance genes among *Enterobacteriaceae* in animals and man. In contrast, others did not find a consistent relationship between plasmid profile and resistance pattern of animal strains and strains isolated from humans in contact with these animals (84,95).

*Meat and meat products*

During slaughtering, carcasses are inevitably contaminated with gut flora, especially when slaughtered under poor hygienic conditions (64,66). Howe *et al.* (45) showed that direct carcass contamination did occur from the rectal contents and from cross contamination from other animals. Shooter *et al.* (96) isolated *E. coli* from floors, instruments and carcasses in an abattoir, showing that bacterial contamination was commonly present.

Shooter *et al.* (96) also investigated meat on arrival in a hospital kitchen and showed *E. coli* contamination. Consequently hands and kitchen surfaces become contaminated with bacteria when the meat is handled. As a result humans may become colonised with resistant *E. coli* after handling uncooked or not properly handled cooked meat (64,66). Handling and ingestion of contaminated raw meat has been suggested as the most important route by which animal *E. coli* strains might reach man (64). In contrast, Guinee *et al.* (37) showed higher percentages of resistant *E. coli* in vegetarians than in meat-eating individuals. This might be due to contamination of vegetables with resistant bacterial strains from the environment (57,88,97).

**In conclusion**

From the literature it is clear that the use of antibiotics by humans themselves is considered to be a major contributing factor to the problem of antibiotic resistance in man (50,51,90,110,114). However, a contribution to this problem by the extensive usage of antibiotics in veterinary medicine cannot be excluded (28,69). Animals carry large numbers of resistant bacteria and each time that animals are treated with antibiotics this could be considered as a contribution to this problem. Moreover, current animal husbandry practices ensure the rapid dissemination of resistant bacteria and resistance genes in a herd.

The potential public health risks associated with veterinary antibiotic use are:

1. selection of resistant micro-organisms under the pressure of antimicrobial therapy in animals, especially the intensively reared animals intended for food production
2. contact with or ingestion of animal products contaminated with enteric bacteria of animal origin harbouring resistance factors which may colonize the human intestinal flora or transfer of resistance plasmids to this flora

To find out if and to what extent resistance genes in the intestinal tract of pigs contribute to the resistance problems encountered in human medicine the present study was designed.

## AIMS OF THE STUDY

As described before, the literature is not clear about the possible public health risk of antibiotic use in veterinary medicine. Animals might be a source of resistance genes for the human intestinal flora, but it is unclear to what extent. The present study was designed to detect if and to what extent, antibiotic resistance found in the human intestinal flora was derived from resistant bacteria from animals. Therefore, three groups of people with different intensity and frequency of contact with pigs or pig products were compared:

A. pig farmers, who have daily intensive contact with pigs and pig faeces, and are regularly exposed to antibiotics used for pig medication
B. abattoir workers, who have daily contact with pigs, pig carcasses or pig meat
C. as a control group (sub)urban residents, who are not in contact with previous mentioned risk factors

Furthermore, pigs living at the same farm as the pig farmers were investigated. The influence of direct contact with animals or animal products as well as recent antibiotic use in human and pig medicine was investigated.

* The prevalence and high degree of antibiotic resistance in the faeces of pigs housed in different compartments at the same farm was monitored during 11 months to study the variation in prevalence of resistance over time in a population of different groups of pigs.
* The prevalence and high degree of resistance to antibiotics commonly used in pig and human medicine as well as resistance patterns of faecal *E. coli* isolated from pigs and humans with different contact with pigs and antibiotics, i.e. pig farmers, abattoir workers and the open population, were determined.
* *In vitro* and *in vivo* transfer experiments with plasmids of porcine and human *E. coli* strains isolated at the same farm were performed.
* Finally, a feasible and reliable method for monitoring antibiotic resistance in pig populations was developed. Faecal samples of pigs living at farms and floor droppings collected from trucks transporting fattening pigs were compared for the prevalence of resistance as well as for antibiotic susceptibility and resistance patterns.

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# MONITORING ANTIBIOTIC RESISTANCE OF *ENTEROBACTERIACEAE* ISOLATED FROM THE FAECAL FLORA OF FATTENING PIGS

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Antibiotic resistance of *Enterobacteriaceae* isolated from the faecal flora of fattening pigs

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SUMMARY

*From June 1991 to April 1992 407 faecal samples were collected from three groups of pigs (I n=248, II n=87, III n=72) at a pig fattening farm to determine the prevalence and the degree of antibiotic resistance of Enterobacteriaceae as well as the antibiotic susceptibility of the strains isolated. Despite the absence of mass-medication during the observation period, the prevalence of resistance to the most commonly used antimicrobial agents in veterinary medicine was high (range amoxycillin 70%-97%, oxytetracycline 89%-100%, sulphamethoxazole 88%-100%, trimethoprim 78%-100%). The high degree of resistance to oxytetracycline and sulphamethoxazole ranged from 8%-67% and 4%-46%, respectively. The percentage of the isolated Escherichia coli strains resistant to oxytetracycline, streptomycin and sulphamethoxazole ranged from 49% to 68%; the other agents tested showed lower percentages (0-13%). Resistance to three or more antibiotics was observed in 43% of the isolates. Of the 52 resistance patterns that could be distinguished, 51% was accounted by only four patterns: oxytetracycline+streptomycin+sulphamethoxazole 20%, sulphamethoxazole 12%, streptomycin+sulphamethoxazole 11% and streptomycin+oxytetracycline 8%.*

## INTRODUCTION

The use of antibiotics in medical and veterinary practice has led to an increase in bacterial resistance in both populations, which might interfere with the effective treatment of bacterial infections (11,14,26). However, the use of antibiotics is not the only reason for the increase in resistance. It is known that resistant bacteria can be transferred in different ways (27). One of them is from animal to humans, but vice versa is also possible (23,28). However, the frequency of transfer of resistant bacteria *in vivo* is difficult to determine (14). It is still debated to what extent the use of antibiotics in veterinary medicine is an important cause of the resistance problem encountered in humans, but it has been shown that antibiotic use in humans is of major importance (6,31). Hospitals are especially breeding places for resistance, and newly admitted patients are a common source of resistant hospital strains. More specially they, or better their bacterial flora, are the barometers of antibiotic use in the community and therefore reflect the frequency of resistance there (22). The same is most likely the case in animal populations. Moreover, as such resistant bacteria represent a constant pool of resistance genes potentially transferable to pathogenic bacteria and indirectly transferable by animal products contaminated by enteric flora to the human intestinal tract, it seems appropriate to monitor this resistance (29,34).

Some studies described the prevalence of resistance in human and in animal enteric flora, but the results are difficult to compare due to differences in geographic locations, time and because of the various methods used to determine antibiotic resistance (3,4,8,12,29). In addition, almost no data are available about the stability of the prevalence of resistance over a certain period of time.

The present study was undertaken to investigate the prevalence of antibiotic resistant *Enterobacteriaceae* in faecal samples of pigs and changes in resistance over time (11 months) on a pig fattening farm. In addition, the degree of resistance and the reproducibility of sampling were determined as well as the antibiotic resistance patterns of the isolated *Escherichia coli*.

## MATERIALS AND METHODS

### Housing conditions of the pigs

The finishing house (±600 pigs) consisted of five compartments, each containing eight pens with ±15 pigs per pen. Pigs were obtained from five breeders and each compart-

ment housed pigs from two different breeders. An "all in, all out" system was used for each compartment. After removal of the pigs for slaughter, the compartments were thoroughly cleaned and disinfected with chloramide[*1].

**Faecal sampling**

Three different groups of pigs were sampled, between June 1991 and April 1992; 407 faecal samples were collected in total. The farm was visited by the same person twelve times to collect the faecal samples, the first four times once a week and then monthly. Each time faecal samples came from the same three (out of five) compartments. On the first seven occasions (period 1-7), six rectal samples (6 out of 15 pigs) from one pen and one floor sample from the other pens were collected from each of the three compartments. More than six rectal faecal samples were collected during the periods 3 and 4 (12 and 2 more, respectively). During the remaining periods (8-12) only floor samples, one from each pen, were taken.

group I (n=248): from June to August 1991 (period 1-6),

group II (n= 87): from October to December 1991 (period 7-9),

group III (n= 72): from February to April 1992 (period 10-12).

**Antibiotic use**

*Growth promotion*

During the sampling period two types of feed-mix were supplied. From arrival until 16 weeks of age the pigs were given pellets containing 40 mg/kg of the growth promoter virginiamycin, an antibiotic which is not active against *Enterobacteriaceae*. Subsequently pellets containing 20 mg/kg of virginiamycin were given until the pigs reached the age of 6 months.

*Therapeutic use*

During the sampling period 15 pigs received parenteral antimicrobial treatment. One pig (period 2: second week) was treated with penicillin[*2] for 6 days. In period 8 two pigs received oxytetracycline[*3] for 6 days and in the periods 10 and 11 oxytetracycline[*3] was used for eight and four pigs respectively. No information about previous antibiotic use could be obtained from the suppliers.

[*1] *Halamid®, Veip bv, Wijk bij Duurstede, The Netherlands.*

[*2] *Duplocillin®, Mycofarm, De Bilt, The Netherlands.*

[*3] *Geomycine®, Dopharma, Raamsdonkveer, The Netherlands.*

**Microbial analysis of the faecal samples**

Faecal samples were transported immediately to the microbiological laboratory where one gram of each sample was diluted in 9 ml of 0.9% sodium chloride solution (in 20 % glycerol) and stored at -20°C until assayed. Samples (37 $\mu$l) of $10^{-2}$ and $10^{-3}$ dilutions in 0.9% sodium chloride solution were then inoculated on Levine-agar (BBL 11221[*4]) plates with and without antibiotics, using a spiral plater[*5]. Antibiotics were used in the agar plates in the following concentrations: amoxycillin (Amx 25 mg/l), ciprofloxacin (Cip 4 mg/l), flumequin (Flu 4 mg/l), neomycin (Ne 8 mg/l), nitrofurantoin (Ft 50 mg/l), oxytetracycline (Ot 25 mg/l), sulphamethoxazole (Smx 100 mg/l), trimethoprim (Tmp 8 mg/l). Apramycin (Ap 32 mg/l) was tested in the periods 8-12. For trimethoprim testing 5% lysed horse blood was added to the agar. These antibiotics were selected because they are regularly used therapeutically in pigs in The Netherlands, except for apramycin, which has not been used on large scale. Amoxycillin is not used commonly in pig medicine, but the *in vitro* activity is the same as that of ampicillin, which is used more extensively. Ciprofloxacin and nitrofurantoin have cross-resistance with enrofloxacin and furazolidon, respectively. Enrofloxacin has not been used on large scale yet on pig farms in The Netherlands, but furazolidon has been used extensively for several decades. Because of the poor solubility of furazolidon nitrofurantoin was tested.

*Escherichia coli* appears on Levine-agar plates as purple colonies with a black centre and a metallic green shine. Only these colonies were counted. The minimum detectable level of bacterial growth, by using the spiral plater, was $10^3$ *Enterobacteriaceae*/g faeces. Other *Enterobacteriaceae*, however, may grow on the plates as well. From each plate with and without antibiotics one colony, which answered the description of *E. coli*, was picked up and tested for growth at 42°C overnight in tryptone water (Oxoid L42[*6]) and with the indole reaction. If both tests were positive the micro-organism was considered to be an *E. coli* and stored in a peptone glycerol (87%) solution (Oxoid CM9/ Merck 4094[*7]) at -20°C. Ninety-four per cent of all colonies tested were indole positive and showed growth at 42°C and were thus considered to be *E. coli*.

[*4] *Becton Dickinson BV, Etten-Leur, The Netherlands.*

[*5] *Model C, Spiral Systems Inc. Salm en Kip, Utrecht, The Netherlands.*

[*6] *PCH Diagnostica, Haarlem, The Netherlands.*

[*7] *Merck Nederland, Amsterdam, The Netherlands.*

*Prevalence of antibiotic resistance*

The prevalence of resistance (%) in the population is given by the number of samples that grew on antibiotic-containing plates divided by the total number of samples tested x100%.

*Degree of antibiotic resistance*

The degree of antibiotic resistance of each faecal sample to each of the agents tested was assessed as described before (5,21). It was calculated as the number of colony forming units (CFU) of resistant *Enterobacteriaceae* divided by the total number of *Enterobacteriaceae*. Two degrees of antibiotic resistance to a particular agent could be distinguished: a low degree, i.e. less than 50% of the faecal *Enterobacteriaceae* flora was resistant to that agent, and a high degree, i.e. 50% or more (thus the majority) of the faecal *Enterobacteriaceae* flora was resistant to that agent.

*Antibiotic susceptibility testing*

The antibiotic susceptibility of the *E. coli* strains isolated from the Levine-agar plates without antibiotics was determined using a microbroth dilution method in Iso-sensitest$^R$ broth (Oxoid CM473) with an inoculum of $5x10^5$ CFU/well.

The antimicrobial agents tested and the breakpoint concentrations for susceptibility according to the guidelines of the Dutch Working Party (16) were as follows: amoxycillin (Amx 16 mg/l), amoxycillin+clavulanic acid (Amc 16 mg/l), chloramphenicol (C 8 mg/l), gentamicin (Gm 4 mg/l), nalidixic acid (Na 8 mg/l), neomycin (Ne 16 mg/l), nitrofurantoin (Ft 32 mg/l), oxytetracycline (Ot 16 mg/l), streptomycin (S 16 mg/l), sulphamethoxazole (Smx 128 mg/l) and trimethoprim (Tmp 2 mg/l). For apramycin (Ap) the breakpoint concentration was 16 mg/l (15). For trimethoprim testing thymidine phosphorylase was added to the Iso-sensitest$^R$ broth. After incubation for 18-24hrs at 37°C, the minimal inhibiting concentration (MIC) was determined as the lowest concentration that inhibited visible growth. *Escherichia coli* strains ATCC 25922 and ATCC 35218 were used as controls for the susceptibility tests.

**Statistical analysis**

The means of the antibiotic prevalence of the different periods within one group were compared by using Student's t-test (significance $P \leq 0.05$). To compare the three different groups of pigs with each other, Student's t-test for the difference of two population means with pooled variance was used (significance $P \leq 0.05$). This last test was also used to compare the means of the rectal and floor faecal samples from the pigs of group I with each other.

The error in the method introduced by making tenfold dilutions and by using the spiral

plater was calculated from the standard error of the mean and was 0.5 $^{10}$log.

## RESULTS

After seven periods of faecal sampling it was found that there were no significant differences between the prevalence of antibiotic resistant *Enterobacteriaceae* in the individual rectal and floor samples (Table 1). Therefore, only floor samples were collected during the periods 8-12. Of the total number of purple colonies with a black centre and a metallic green shine, 94% were indole positive and showed growth at 42°C and were identified as *E. coli*. No resistance to any of the antimicrobial agents tested was observed in 4 of the 407 faecal samples (1%).

### *Prevalence of antibiotic resistance*

The prevalence of resistant *Enterobacteriaceae* was compared per antibiotic as a function of time. The prevalence of resistance (%) to oxytetracycline over time in the different groups is shown in Figure 1. The prevalence of resistance (%+SD) to the other compounds is given in Table 2.

A high prevalence percentage of resistance to oxytetracycline, sulphamethoxazole, amoxycillin and trimethoprim was found. Resistance to ciprofloxacin was not observed.

*Table 1: Prevalence of resistance (%) of rectal and floor faecal samples from the pigs of group 1 (n=248).*

| antimicrobial agent | **rectal samples** n=122 | | | **floor samples** n=126 | | |
|---|---|---|---|---|---|---|
| | X | SD | range | X | SD | range |
| Amx | 69 | 15 | (50-87) | 71 | 17 | (38-81) |
| Cip | 0 | 0 | | 0 | 0 | |
| Flu | 1 | 2 | (0-6) | 0 | 0 | |
| Ft | 7 | 16 | (0-40) | 10 | 12 | (0-29) |
| Ne | 36 | 24 | (20-83) | 36 | 16 | (14-62) |
| Ot | 86 | 16 | (67-100) | 91 | 13 | (67-100) |
| Smx | 86 | 13 | (72-100) | 89 | 18 | (52-100) |
| Tmp | 90 | 20 | (50-100) | 86 | 25 | (38-100) |

*X= mean, SD= standard deviation; Amx= amoxycillin, Cip= ciprofloxacin, Flu= flumequin, Ne= neomycin, Ft= nitrofurantoin, Ot= oxytetracycline, Smx= sulphamethoxazole, Tmp= trimethoprim.*

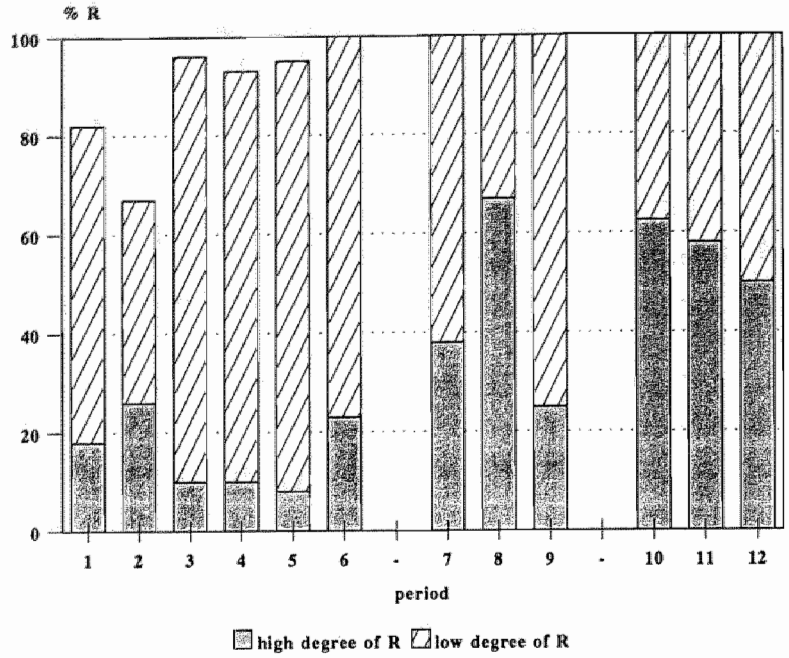


*Fig.1: Prevalence of resistance (%) to oxytetracycline of Enterobacteriaceae isolated from three groups of pigs as a function of time. Also shown are the frequencies (%) of low degree (<50%) and high degree (≥50%) of resistance. Group I: periods 1-6, II: 7-9, III: 10-12; %R= resistance percentage.*

One sample showed resistance to flumequin and another to apramycin. The prevalence of resistance to nitrofurantoin was low (3-14%).

Within the three groups no significant differences were observed between the samples taken at different times, except for group I in the periods 2 and 4. During period 2 the prevalence of resistance to neomycin was higher (i.e. 72%, X=mean 36%) and for the other antibiotics significantly lower than during the other periods (Amx 44%, X=70%; Ft 3%, X=9%; Ot 67%, X=89%; Smx 62%, X=88%; Tmp 44%, X=88%). During period 4 only a significant difference was observed for nitrofurantoin (34%, X=9%). Between the groups there were only significant differences between group I and III for amoxycillin and between group I and II, II and III for neomycin (Table 2).

*Degree of antibiotic resistance*

The degree of resistance (%) to oxytetracycline over time in the different groups is shown in Figure 1. A high degree of resistance to oxytetracycline and sulphamethoxazole was present in all periods tested (Table 3). The percentage ranged from 8% to 67% for oxytetracycline in periods 5 and 8, respectively. For sulphamethoxazole the percentage

*Table 2: Prevalence of resistance (%) of faecal Enterobacteriaceae isolated from three groups of pigs.*

| group: | I n=248 | | | II n=87 | | | III n=72 | | |
|---|---|---|---|---|---|---|---|---|---|
| | X | SD | range | X | SD | range | X | SD | range |
| Amx | 70 | 15[a] | (44-84) | 88 | 15 | (71-97) | 97 | 2[a] | (96-100) |
| Cip | 0 | 0 | | 0 | 0 | | 0 | 0 | |
| Flu | 0.4 | 1 | (0-3) | 0 | 0 | | 0 | 0 | |
| Ft | 9 | 13 | (0-34) | 3 | 5 | (0-8) | 14 | 21 | (0-38) |
| Ne | 36 | 19[b] | (21-72) | 83 | 11[bc] | (71-93) | 50 | 18[c] | (38-71) |
| Ot | 89 | 12 | (67-100) | 100 | 0 | | 100 | 0 | |
| Smx | 88 | 14 | (62-100) | 100 | 0 | | 100 | 0 | |
| Tmp | 88 | 22 | (44-100) | 100 | 0 | | 78 | 16 | (67-96) |
| Ap | -- | -- | | 0 | 0 | | 1 | 2 | (0-4) |

*Legend see Table 1; -- = not tested, Ap = apramycin; [a], [b], [c] = significant different ($P \leq 0.05$).*

*Table 3: High degree of resistance (%) of faecal Enterobacteriaceae isolated from three groups of pigs over the study period.*

| group: | I n=248 | | | II n=87 | | | III n=72 | | |
|---|---|---|---|---|---|---|---|---|---|
| | X | SD | range | X | SD | range | X | SD | range |
| Amx | 18 | 13 | (0-36) | 0 | 0 | | 6 | 5 | (0-8) |
| Cip | 0 | 0 | | 0 | 0 | | 0 | 0 | |
| Flu | 0 | 0 | | 0 | 0 | | 0 | 0 | |
| Ft | 0 | 0 | | 0 | 0 | | 0 | 0 | |
| Ne | 3 | 6 | (0-15) | 0 | 0 | | 0 | 0 | |
| Ot | 16 | 8[ab] | (8-26) | 43 | 21[a] | (25-67) | 51 | 11[b] | (42-63) |
| Smx | 12 | 6 | (6-23) | 19 | 15 | (4-33) | 26 | 19 | (8-46) |
| Tmp | 5 | 4 | (0-10) | 1 | 2 | (0-3) | 0 | 0 | |
| Ap | 0 | 0 | | 0 | 0 | | 0 | 0 | |

*X = mean, SD = standard deviation; [a], [b] = significant different ($P \leq 0.05$). Amx = amoxycillin, Cip = ciprofloxacin, Flu = flumequin, Ft = nitrofurantoin, Ne = neomycin, Ot = oxytetracycline, Smx = sulphamethoxazole, Tmp = trimethoprim, Ap = apramycin.*

ranged from 4% (period 9) to 46% (period 11). The range for amoxycillin was between 0% and 36% (period 1) and for trimethoprim between 0% and 10% (period 2). A high degree of resistance was not found for nitrofurantoin, apramycin or flumequin. Only in period 2 was a high degree of resistance observed in 15% of the samples resistant to neomycin.

Significant differences between the faecal samples of one group of pigs taken at different times were found for group I, in period 1 for amoxycillin (28%, X=mean 18%), sulphamethoxazole (23%, X=12%) and in period 6 for trimethoprim (0%, X=5%). In period 2 there were significant differences for amoxycillin (0%, X=18%), neomycin (15%, X=3%), oxytetracycline (26%, X=16%) and trimethoprim (10%, X=5%). A significant difference between the three groups of pigs was noticed for oxytetracycline between group I and II and between I and III (Table 3).

*Antibiotic susceptibility*

The antibiotic susceptibility was determined for 387 out of 407 faecal samples. Eleven *Enterobacteriaceae* showed no growth at 42°C and were indole negative, and nine *E. coli*

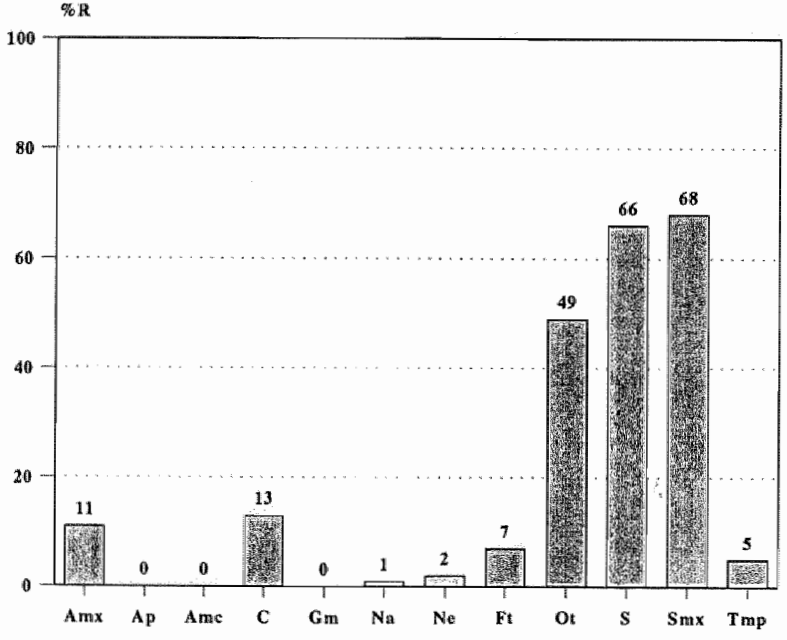


*Fig.2: Antibiotic resistance (%) of E. coli isolated from pigs (n=387). Amx= amoxycillin, Ap= apramycin, Amc= amoxycillin + clavulanic acid, C= chloramphenicol, Gm= gentamicin, Na= nalidixic acid, Ne= neomycin, Ft= nitrofurantoin, Ot= oxytetracycline, S= streptomycin, Smx= sulphamethoxazole, Tmp= trimethoprim; %R= resistance percentage.*

isolates were overgrown by *Bacillus* spp. Forty (10%) of the 387 isolates were susceptible to all the antibiotics tested. Resistance to oxytetracycline, streptomycin and sulphamethoxazole was found in 188 (49%), 257 (66%) and 263 (68%) strains respectively (Figure 2). Distinctly lower percentages of resistance were observed for the other agents tested, i.e. amoxycillin 11%, chloramphenicol 13%, neomycin 2%, nalidixic acid 1%, nitrofurantoin 7% and trimethoprim 5%. No resistance to apramycin, amoxycillin+clavulanic acid or gentamicin was found (Figure 2).

Eighty-five faecal isolates (22%) were resistant to only one antibiotic and 94 (24%) to two, 112 (29%) to three, 37 (10%) to four, 13 (3%) to five, and 5 (1%) to six antibiotics. Only one isolate was resistant to 7 of the 12 antibiotics tested. Overall, 52 different resistance patterns were observed. The combination of oxytetracycline + streptomycin + sulphamethoxazole (n=76, 20%) was most frequently observed, followed by sulphamethoxazole alone (n=46, 12%) and in combination with streptomycin (n=42, 11%). Resistance to the combination streptomycin and oxytetracycline was found in 32 strains (8%), resistance to amoxycillin in combination with oxytetracycline, streptomycin and sulphamethoxazole was observed in 11 strains (3%).

## DISCUSSION

The results of this study showed a high prevalence of resistance to oxytetracycline and sulphamethoxazole in the absence of recent antibiotic therapy during the stay at this farm. This is in accordance with the results of other studies (13,23,35). It is very likely that the resistant strains found in this study were already present in the faecal flora of the pigs when they arrived at the farm, probably due to transfer from the faecal flora of the sow to the piglets and perhaps also because of antimicrobial therapy before weaning.

In this study a high degree of antibiotic resistance was observed for the most commonly used antibiotics in veterinary medicine (amoxycillin, oxytetracycline, sulphamethoxazole, trimethoprim) and also for neomycin. Except for oxytetracycline the high degree of resistance was constant over time. A high degree of resistance means that the majority of faecal *Enterobacteriaceae* are resistant to the antibiotic tested and can be considered to be a major reservoir of resistance genes (21). In humans, a high degree of resistance of *Enterobacteriaceae* in the faecal flora of individuals was also found to be constant over time (24).

In this study minor differences were observed between different groups of pigs, pigs from

different breeders and between pigs from different compartments. The data confirm the hypothesis that resistance in a population against a particular antibiotic is relatively constant and more related to the amounts of antibiotics used previously than currently (20,23). However, this does not mean that these antibiotics are no longer useful. In pigs oxytetracycline is mainly used for the treatment of respiratory tract infections and still seems very effective. Trimethoprim, used for respiratory and gastrointestinal infections, is still an effective agent, especially in combination with sulphonamides.

Langlois *et al.*(18) showed that the use of antibiotics is not the only factor that influences the prevalence of resistant bacteria. The age of the animals studied and the housing conditions should also be taken into account. The highest resistance percentages for ampicillin, streptomycin, sulphamethoxazole and tetracycline were observed in pigs aged 6 months or younger. High resistance to carbenicillin, which has never been used on a large scale in pigs, was also observed (18). The age of the pigs and the housing conditions in intensive pig husbandry, which enforces high faecal contact, are probably important factors in the present study as well.

Veterinary and human use of quinolones for the treatment of bacterial infections has given rise to resistant bacterial strains (2,9,25). In recent years new fluoroquinolones (ciprofloxacin, norfloxacin, enrofloxacin) have become available for clinical use. Enrofloxacin is only registered for use in veterinary medicine and is not available for human therapy. It is, however, closely related to and shows complete cross-resistance with ciprofloxacin (19). Both new agents have proved their usefulness in treating infections caused by bacteria resistant to the older quinolones (9,26). However, after a few years of clinical use an increase in resistance was observed in poultry as well as in human isolates; the increase was more marked for *Campylobacter* spp. than for the *Enterobacteriaceae* (10,30). It has been suggested that especially the veterinary use of quinolones could lead to an increase in the bacterial resistance of human pathogens (1). However, in this study ciprofloxacin-resistant *E.coli* strains were not isolated and only one faecal sample (1/407) showed resistance to flumequin.

Neomycin is mainly used in young pigs for the treatment of diarrhoea. The differences found for neomycin resistance are most likely related to the use of this drug by the breeders. As mentioned earlier this could not be confirmed because information about antibiotic use could not be obtained from the pig breeding farms.

Apramycin is an aminocyclitol antibiotic registered for use in animals only. Apramycin is rarely used in veterinary practice in The Netherlands. Because of the cross-resistance between apramycin and gentamicin, it has been suggested that the use of apramycin in animals may lead to gentamicin-resistant isolates in hospital patients (7). Several studies

described the transfer of resistance to apramycin between bacteria from animals to human gut flora (15,33,36). In this study apramycin-resistant faecal strains were isolated in only 1 out of the 120 samples tested. It is not likely that this low resistance to apramycin will be a risk factor for gentamicin resistance in humans.

The percentage susceptibility described in the present study was similar to that obtained for a group of healthy pigs described by Wray (35). Similar percentages (except for S and Smx) were observed by Langlois *et al.* (17), who investigated a herd of pigs which had not received antibiotics in 13 months prior to the study (Table 4). The fact that different microbiological methods were used in these studies cannot explain the differences found in resistance to some antibiotics.

The results of this study showed high resistance to drugs commonly used in veterinary medicine, i.e. oxytetracycline and sulphamethoxazole. The high percentage of streptomycin resistance can be explained by the possible carriage of drug resistance on the same plasmid as that for oxytetracycline and sulphamethoxazole. Other investigators have also found this antibiotic resistance profile (29,32).

Although in recent years the use of chloramphenicol in pigs has been forbidden in The Netherlands, 13 % of the *E. coli* strains were resistant to this drug. This is most likely due to a common plasmid which encodes for Smx+S or for Smx+S+Ot, in addition to chloramphenicol. In this study only 2 out of 50 chloramphenicol-resistant faecal isolates showed single resistance to chloramphenicol. The majority (48 out of 50) of the chloramphenicol resistant isolates also showed resistance to one or more other antibiotics, especially sulphamethoxazole, oxytetracycline and streptomycin. Saida *et al.* (32) also described resistance to combinations of chloramphenicol with Smx+S or Smx+S+Ot in

*Table 4: Antibiotic resistance (%) in different studies.*

| antibiotic agent | Amx | C | Ne | Ot | S | Smx |
|---|---|---|---|---|---|---|
| present study 1991-1992 | 11 | 13 | 2 | 49 | 66 | 68 |
| Langlois (17) 1988 | 9.2 | 0.8 | 0.8 | 59.1 | 34.5 | 15.6 |
| Wray (35) 1983 | 10 | 0 | 2 | 34 | 70 | 86 |

*Amx= amoxycillin, C= chloramphenicol, Ne= neomycin, Ot= oxytetracycline, S= streptomycin, Smx= sulphamethoxazole.*

1% and 22.2% of the pigs, respectively.

Remarkably, the resistance to furazolidon, which has been used extensively for many years, was relatively low (7%).

## CONCLUSION

The results of this study suggest that for resistance surveillance a single collection of floor faecal samples in several compartments of pig fattening farms gives a reliable indication of the prevalence and the degree of antibiotic resistance of *Enterobacteriaceae* in these pig fattening units. In addition the prevalence and the degree of resistance are relatively stable over an extended period of time (in this study 11 months) in the absence of mass-medication. Thus, regular monitoring once a year seems sufficient to detect major changes in the prevalence and degree of antibiotic resistance of *Enterobacteriaceae* of the intestinal flora of fattening pigs.

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# RESISTANCE IN FAECAL *ESCHERICHIA COLI* ISOLATED FROM PIG FARMERS AND ABATTOIR WORKERS

Nijsten R., London N., Bogaard v.d. A., Stobberingh E.
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*om three populations of healthy adult volunteers (290 pig ers, 160 (sub)urban residents) living in the South of The for the prevalence and degree of antibiotic resistance of*

*revalence of resistance to amoxycillin, neomycin, oxytetra- nd trimethoprim were observed. The pig farmers showed the ance and the (sub)urban residents the lowest. In contrast no ı degrees of resistance were observed, except for neomycin. ıd abattoir workers have regular contact with pigs differences were observed. However, because abattoir workers with ›ig(carcass) contact did not show significant differences, this ›rtant source of resistant E. coli in pig farmers. pig farmers (5%) and abattoir workers (8%) than by (sub)ur- esult in significantly different resistance percentages.*

## INTRODUCTION

Since the introduction of antimicrobial agents these have been successfully used to prevent and treat bacterial diseases in man and other species. The availability of antibiotics means that many previously severe infections can now be treated. In addition, antibiotics are used for growth promotion in animal husbandry and in agriculture for crop protection. As antibiotics are not only very effective, but also remarkably safe drugs this safety may have provoked liberal even lavish use in man and other animals. The use of antibiotics, however, leads inevitably to emergence of resistance in the endogenous bacterial flora of treated persons and animals alike, against the antibiotics used or to other drugs (4,18). These enteric micro-organisms may colonize other persons or animals and transfer resistance plasmids to their faecal flora. Consequently, the environmental bacterial population may be contaminated after faeces excretion. Lester *et al.* (9) showed that persons with a few resistant bacteria in their intestinal flora will have more chance of developing an infection with resistant bacteria after antibiotic therapy than persons with no resistant strains at all.

Many studies have examined the resistance of enteric bacteria in humans after antibiotic therapy (1,6,8,17,25), but there is much less information available on the prevalence of antibiotic resistance in the faecal flora of healthy adults who have not used antibiotics recently (2,3,5,13,16). However, such subjects are potential recipients of antimicrobial agents. Farm workers can directly become colonized by resistant bacteria due to close contact with animals and their faeces (10,11,21), but are also directly exposed to antibiotics used for treatment or prevention of diseases in animals (12). Abattoir workers have daily contact with contaminated carcasses and gut contents (7,14,15,26). A common risk factor for colonization with resistant microorganisms in all three groups is personal use of antibiotics.

To elucidate the importance of spread of resistance from food animals to man we studied in the same region the antibiotic resistance in three populations with different risks of exposure to faecal bacteria from pigs, i.e. pig farmers, abattoir workers and as a control group, (sub)urban residents. As the faecal flora is considered the most important reservoir of resistant micro-organisms and the antibiotic resistance of this flora is an indicator for the resistance of potentially pathogenic bacteria in a population (8,9,13,28), faecal samples of these three populations were analyzed for the prevalence and degree of antibiotic resistance of *E. coli*.

**Collection of the faecal samples**

Faecal samples, one from each person, were received from adult pig farmers (290), pig abattoir workers (316, of which 73 were meat-inspectors) and (sub)urban residents (160) all living in the same area. After receipt, the samples were diluted ($10^{-1}$) in physiological saline, containing 20% (v/v) glycerol and stored at -20°C until examined. All participants were asked to answer a questionnaire concerning antibiotic use in the previous three months. Additional information about recent hospital stay and previous antibiotic use by family members was obtained from the pig farmers and abattoir workers. The abattoir workers were also asked to give some information about keeping domestic animals or pigs and their daily duties at the slaughterhouse.

**Bacteriological analysis of the faecal samples**

The methods used to determine the prevalence and degree of resistance were as described before (19). In brief, after thawing the samples ($10^{-1}$), tenfold dilutions ($10^{-2}$-$10^{-5}$) in physiological saline were made. Thirty-seven $\mu$l of these dilutions were inoculated with a spiral plater on Levine-agar plates (BBL 11221, 27), a selective medium for *E. coli*, with and without antibiotics. The antibiotic concentrations (Table 1) were based on NCCLS guidelines and modified where appropriate so that the data was comparable with that of previous studies (2,3). Only colonies with the appearance of *E. coli* (i.e. purple with a black centre and a metallic green shine) were counted. The total number and the number of resistant *E. coli* were determined. The minimum detection level of bacterial growth was $10^3$ colony forming units (CFU)/g faeces. From each agar plate without antibiotics one colony with the appearance of *E. coli* was picked and tested for growth at 42°C overnight in tryptone water (Oxoid L42) and for the indole reaction. If these tests were positive the micro-organism was considered to be *E. coli*. For the first 50 isolates this identification was confirmed with Api-20E test (BioMerieux, Den Bosch, The Netherlands).

*Prevalence of antibiotic resistance*

The prevalence of antibiotic resistance was defined as the percentage of faecal samples showing any growth of *E. coli* on antibiotic-containing plates.

*Degree of antibiotic resistance*

The degree of resistance of each sample was calculated as the percentage of the total number of colonies that was resistant. Two degrees of antibiotic resistance to a particular antimicrobial agent were distinguished (3,13), namely the proportion of faecal samples

with a ratio $<50\%$ was defined as low degree of resistance, and the proportion of faecal samples with a ratio $\geq 50\%$ was defined as high degree of resistance (thus the majority of the strains showing resistance to that agent).

The antimicrobial agents used in this study were selected because these or closely related compounds are regularly used for the treatment of humans and pigs in The Netherlands, except apramycin which is only registered for use in animals but is not extensively used in The Netherlands.

**Statistical analysis**

In the analysis of the differences in prevalence and degree of antibiotic resistance of the faecal samples of the three populations a Chi Square test with continuity correction was performed. A Fisher Exact test was used if the expected frequency in at least one cell was 5 or less. A two-sided significance level of $\leq 0.05$ was used.

Multiple logistic regression was used to analyze the contribution of the origin of the three study populations (independent variable) to the prevalence of resistance (dependent variable) to a particular antibiotic. The antibiotics other than the dependent variable were considered to be independent variables simultaneously, a two-sided significance level of $\leq 0.05$ was used.

The error of the method by using the spiral plater and by making tenfold dilutions, calculated from the standard error of the mean, was 0.5 $^{10}$log.

## RESULTS

Ninety-five per cent of the pig farmer colonies, 94% of the abattoir workers colonies and 92% of the (sub)urban residents colonies that grew on Levine-agar plates showing the morphology typical of *E. coli* were identified as *E. coli*. The other colonies tested were also *Enterobacteriaceae*: *Klebsiella* spp., *Citrobacter* spp., *Enterobacter* spp. and *Proteus* spp. Finally 278 samples of pig farmers, 289 of abattoir workers and 150 of (sub)urban residents were included in the analysis. The other samples failed to grow on the agar plates without antibiotics.

Antibiotic use was recorded by 15 pig farmers and 17 family members. Two farmers had been hospitalized recently. Twenty-five abattoir workers and 25 family members recorded antibiotic use, 5 abattoir workers had been hospitalized recently. By the (sub)urban residents no antibiotic use in the three previous months was recorded. Intensive contact

*Table 1. Prevalence and high degree of antibiotic resistant Escherichia coli (%)*

| Antibiotics | | Prevalence | | | High degree | | |
|---|---|---|---|---|---|---|---|
| | | PF | AW | UR | PF | AW | UR |
| mg/l | | n=278 | n=289 | n=150 | n=278 | n=289 | n=150 |
| Amx | (25) | 62 | 42[a] | 47[bd] | 7 | 9 | 13 |
| Ap | (32)** | 3 | 1 | nt | 0 | 0 | nt |
| Cip | (4) | 1 | 0 | 0 | 0 | 0 | 0 |
| Na | (32) | 5 | 3 | 1 | 1 | 0 | 0 |
| Ne | (8) | 66 | 36[a] | 25[bd] | 7 | 2[a] | 2[bd] |
| Ft | (50) | 8 | 4 | 3 | 0 | 0 | 0 |
| Ot | (25) | 79 | 47[a] | 36[bcd] | 10 | 15 | 8 |
| Smx | (100) | 84 | 45[a] | 40[bd] | 17 | 13 | 10 |
| Tmp | (8) | 53 | 23[a] | 15[bd] | 4 | 4 | 3 |

*Amx= amoxycillin, Ap= apramycin, Cip= ciprofloxacin, Na= nalidixic acid, Ne= neomycin, Ft= nitrofurantoin, Ot= oxytetracycline, Smx= sulphamethoxazole, Tmp= trimethoprim. **= Apramycin was only tested for the slaughterhouse workers and the last 116 farmers faecal samples. PF= pig farmers, AW= abattoir workers, UR= (sub)urban residents; nt= not tested. Significantly different ($P \leq 0.05$): PF and AW: [a], PF and UR: [b], AW and UR: [c]; PF and AW and UR [d]*

with pigs or pig carcasses was recorded by 182 abattoir workers, whereas 104 workers had other duties as well or no direct contact. No information about contact with pigs or pig carcasses was obtained from the remaining abattoir workers (n=30). Fifty-two per cent of the abattoir workers did keep at least one domestic animal, whereas only three persons kept pigs.

*Prevalence of antibiotic resistance*

The prevalence and high degree of resistance are shown in Table 1 and Figure 1. The most significant differences were noticed between pig farmers and (sub)urban residents. The highest prevalence percentages were found for the pig farmers and the lowest for the (sub)urban residents. The highest percentages (i.e. 47%) in the abattoir workers and in the (sub)urban residents group were recorded for oxytetracycline and amoxycillin, respectively, and in the pig farmer group for sulphamethoxazole (84%). Further analysis as to the patterns of prevalence of resistance to amoxycillin, neomycin, oxytetracycline and trimethoprim of *E. coli* isolated from the three populations studied clearly showed that the highest percentage of fully susceptible strains (34%) as well as the lowest percentage of completely resistant isolates (4%) were found in the (sub)urban residents. The converse was observed for the pig farmers.

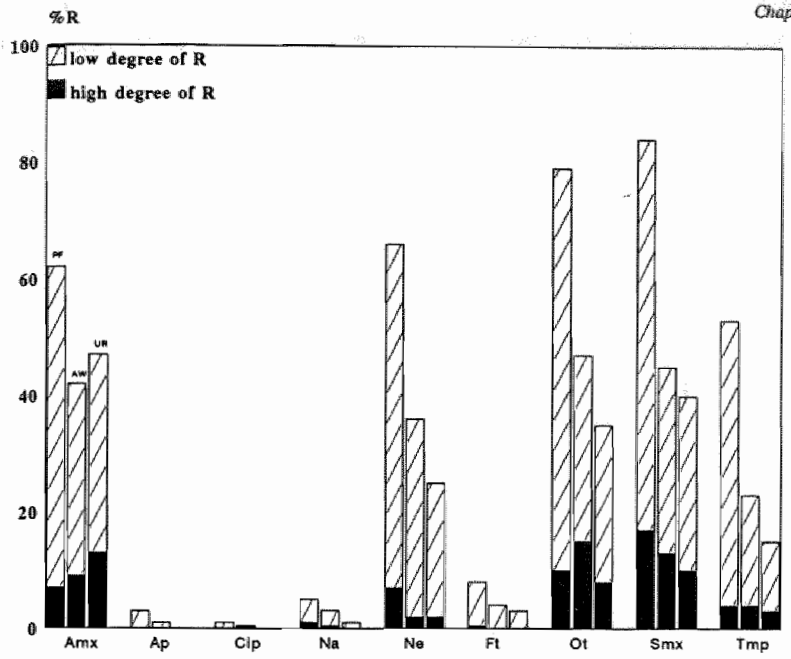


*Figure 1: Prevalence of antibiotic resistance (%) of Escherichia coli isolated from pig farmers (PF, first bar per antibiotic), abattoir workers (AW, second bar) and (sub)urban residents (UR, third bar). Also shown are the proportions (%) of low degree (<50%) and high degree (≥50%) of resistance. Amx= amoxycillin, Ap= apramycin, Cip= ciprofloxacin, Na= nalidixic acid, Ne= neomycin, Ft= nitrofurantoin, Ot= oxytetracycline, Smx= sulphamethoxazole, Tmp= trimethoprim. %R= resistance percentage.*

Logistic regression analysis was performed to estimate the relative risk of prognostic and risk factors (i.e. antibiotics used and population groups) with regard to the (sub)urban residents. The odds ratio (OR), with the 95% confidence interval (CI), for resistance to a particular antibiotic under consistent circumstances was calculated. Both pig farmers (OR 0.4, CI 0.2-0.6) and abattoir workers (OR 0.5, CI 0.3-0.9) showed a low odds ratio for amoxycillin resistance. The pig farmers showed a high odds ratio for neomycin (OR 3.6, CI 2.5-5.4), sulphamethoxazole (OR 6.5, CI 4.0-10.6) and trimethoprim (OR 2.1, CI 1.4-2.9). Resistance to oxytetracycline appeared to be independent of the population tested. For the other antibiotics tested no significant prognostic and risk factors were found.

*Degree of antibiotic resistance*

As presented in Figure 1 all three populations showed, except for neomycin, similar percentages for high degree of resistance, but distinct variations in low degree of resistance. The prevalence and degree of resistance of the meat-inspector samples were not

significantly different from those of the abattoir workers. In addition, no differences in prevalence and degree of resistance were observed between abattoir workers with and without domestic animals. Because only three abattoir workers kept pigs no conclusions about the influence of regular contact with pigs could be drawn.

No significant differences could be observed between abattoir workers with intensive and those without or with less intensive contact with pig faecal contents or pig carcasses.

No significantly different prevalence or degree of resistance rates were observed for those people who had recently used antibiotics compared with those who had not used antibiotics recently (pig farmers 5%, abattoir workers 8%). Nor were differences observed for those recording recent hospital stay (pig farmers 1%, abattoir workers 2%) or antibiotic use by family members (pig farmers 6%, abattoir workers 8%) when compared with those who did not record these factors.

## DISCUSSION

The present study showed significant differences in prevalence of resistance between pig farmers and (sub)urban residents for antibiotics extensively used in human and veterinary medicine in The Netherlands (20). In contrast, the prevalence of the high degree of resistance was, except for neomycin, not significantly different. Several investigators have also observed differences in resistance of the faecal flora of pig farmers / abattoir workers and urban residents (16,22,24) suggesting that contact with livestock was one route by which antibiotic resistance entered the human gut flora. In contrast, Levy *et al.* (13) found no significant difference between rural and urban residents. The general trend in their study was for lower numbers of resistant bacteria to be found in rural samples.

Remarkably, in the present study 15 (5%) pig farmers and 25 (8%) abattoir workers used antibiotics during the three months previous to faecal sampling, whereas none of the (sub)urban residents mentioned recent antibiotic use. This might be an indication that people in contact with pigs or pig carcasses have a greater risk of bacterial infections. A recent study about occupational risk factors for pig farmers showed that pig farmers often suffer from chronic a-specific respiratory tract afflictions, because of regular exposure in pig stables to dust containing fungi, endotoxins, disinfectants etc. (23). This exposure results in a higher probability of respiratory tract infections, which could explain the relatively high percentage of antibiotic usage among the pig farmers. Unfortunately, no information about the reasons of antibiotic therapy was obtained. Moreover, in one of the slaughterhouses studied, each month 4% of the workers were treated with antibiotics for

wounds or eczema (personal communication). Because the control group of (sub)urban residents did not mention recent use of antibiotics, this therapeutic use among pig farmers and abattoir workers might explain the higher prevalence of resistance in these groups than in the (sub)urban residents. The relatively low numbers of pig farmers (n=15) and abattoir workers (n=25) who mentioned antibiotic use could not explain the observed differences between the three populations. Also recent use of antibiotics by family members appeared to be of no influence on antibiotic resistance in this study.

Contact with pigs, pig carcasses or pig faeces might be a possible reason for the differences in prevalence of resistance observed between on one hand pig farmers and abattoir workers and on the other hand the (sub)urban residents. Although no information about the professions of the last group was obtained, it is to be expected that they do not have regular direct contact with pigs. However, no significant differences were observed between the abattoir workers with intensive and those with less intensive pig contact. Therefore, other factors such as more intensive faecal contact, less personal hygiene and protection taken by farmers as compared to abattoir workers might have contributed to these differences. Moreover, it is very likely that direct contact with antibiotics used for treatment of pigs is an additional risk factor for emergence of resistance and selection of resistant strains in the intestinal flora of pig farmers. The results of the logistic regression analysis underscore these suggestions.

Remarkably, significantly different prevalences and high degrees of resistance to neomycin were observed for the pig farmers. Because neomycin is seldom used orally and never parenterally in human medicine but frequently in pigs, the suggestion seems likely that it is not human but mainly veterinary use of neomycin that is responsible for a higher prevalence and high degree of resistance in pig farmers.

This study showed significant differences in the prevalence of antibiotic resistance in the faecal flora of the three populations tested. Direct contact with pigs and pig carcasses may contribute to antibiotic resistance in pig farmers and abattoir workers, in addition to common risk factors such as personal use of antibiotics. Moreover, it is likely that direct contact with antibiotics in medicated pig feed, i.e. mass-medication, influences the prevalence of antibiotic resistance in pig farmers. In the present study only prevalence and degree of resistance were determined. Comparison of plasmids and transfer experiments between pig and human isolates may elucidate the mechanisms involved.

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# ANTIBIOTIC RESISTANCE AMONG *ESCHERICHIA COLI* ISOLATED FROM FAECAL SAMPLES OF PIG FARMERS AND PIGS

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SUMMARY

*The prevalence and degree of antibiotic resistance of 290 faecal samples of pig farmers and 291 faecal samples of pigs were determined. Significantly higher prevalence and high degree of resistance percentages of Escherichia coli were observed in the pig samples. Moreover, the porcine E. coli isolates showed significantly higher resistance percentages for chloramphenicol, nitrofurantoin, oxytetracycline, streptomycin and sulphamethoxazole than the farmer strains. The pig isolates were mainly resistant to oxytetracycline-streptomycin and oxytetracycline-streptomycin-sulphamethoxazole and the farmer isolates to amoxycillin, sulphamethoxazole and streptomycin-sulphamethoxazole. Comparing the resistance patterns of the farmer E. coli isolates with those of the pigs from the same farm (n=259) only 4% of both isolates showed resistance to the same antibiotics.*

*The results of the present study strongly suggested that the resistance in the faecal flora of the pig farmers and their pigs is distinctly different.*

## INTRODUCTION

Antibiotics are extensively used for bacterial infections in human and veterinary medicine. This use selects for resistant micro-organisms which may transfer resistance factors to other bacteria and may contribute to the enhancement and spread of these micro-organisms (18). Transfer of resistant bacteria has been described between different animal species from human to human, from animals to humans and vice versa (10,16,20,21,22, 28,30,31,32).

In contrast to humans and pets, pigs are often treated as a group (mass-medication). Pigs, like other animals, medicated with antibiotics may select resistant *Escherichia coli* in their faecal flora which may transfer their resistance plasmids to other (pathogenic) bacteria (e.g. *Salmonella* spp.) (17,32,34). Moreover pigs have intensive faecal contact and show coprophagy, so by this route they are also continuously exposed to contamination with faecal bacteria and antibiotic resistance genes. People in close contact with animals or using animal products may become colonized with these resistant bacteria from animal intestinal flora. Alternatively, their endogenous flora might become resistant by transfer of plasmids from bacteria of animal origin that pass through their intestinal tract (10,11, 21,33).

In a previous study it was observed that pig farmers, compared to abattoir workers and (sub)urban residents, showed the highest prevalence of resistance (27). Several other factors than direct contact with pigs and pig faeces, like exposure to antibiotics used for treatment of pigs and personal use of antibiotics were suggested to be of influence on the prevalence of resistance in pig farmers. If contamination with intestinal pig flora is important the same resistance levels and comparable resistance patterns could be expected in faeces samples of pig farmers compared to samples from their pigs. However, until now no data are available to confirm or reject this hypothesis. Therefore, the present study was undertaken to compare the prevalence and the degree of antibiotic resistance of *E. coli* in the faecal flora of nearly 300 pig farmers (27) and of the pig population living at their farms. In addition, the antibiotic resistance patterns of *E. coli* strains isolated from both groups were compared.

**Collection of the faecal samples**

Pig farmers in the South of The Netherlands were asked to collect, in small plastic containers, one fresh faecal sample from themselves and one mixed faecal sample from three randomly chosen pigs. Faeces were preferably to be collected from mature gilts and/or heavy porkers. In addition, the farmers were asked to fill in a questionnaire about recent hospital stay, recent use of antimicrobial agents for mass-medication in pigs and antibiotic use by the farmer or his family during the three months preceding the sample collection. The faecal samples and the questionnaire were, immediately after collection, sent to the bacteriological laboratory where at the same day they were diluted ($10^{-1}$) in 0.9% saline, containing 20% (v/v) glycerol and stored frozen at -20°C until examined. Samples obtained 72 hours or more after collection were excluded.

**Bacteriological analysis of the faecal samples**

The methods used to determine the prevalence, the degree of resistance and the susceptibility were as described before (26,27). The *prevalence of antibiotic resistance (%)* in the population was defined as the number of faecal samples that showed growth of *E. coli* on the antibiotic containing agar plates divided by the total number of samples tested multiplied by 100%. The prevalence of resistance to a particular antimicrobial agent is the sum of the low and the high *degree of antibiotic resistance* (3,19). The low degree of antibiotic resistance is the prevalence (%) of faecal samples for which $<50\%$ of the *E. coli* showed resistance to a particular antibiotic. The high degree of antibiotic resistance is the prevalence (%) of faecal samples for which $\geq 50\%$ of the *E. coli* showed resistance to a particular antibiotic (thus the majority of the strains showing resistance to that agent).

The *antibiotic susceptibility* of the *E. coli* strains, isolated from the faeces samples on Levine-agar plates (BBL 11221) without antibiotics, was determined using a microbroth dilution method in Iso-Sensitest$^{R}$ broth (Oxoid CM473) with an inoculum of approximately $5x10^{5}$ CFU/well obtained by dilution of an overnight culture. The breakpoint concentrations used for determining susceptibility are shown in Table 2. *E. coli* isolates showing resistance to nalidixic acid were further tested for resistance to flumequin (4 mg/l) (24) and ciprofloxacin (2 mg/l). The *E. coli* strains ATCC 25922 and ATCC 35218 were used as controls each time susceptibility testing was performed.

The antimicrobial agents tested in this study were selected because these or closely related antibiotics are regularly used in human and/or pig medicine in The Netherlands, except for apramycin which is only used in animals but not on a large scale.

**Statistical analysis**

In the analysis of the differences in prevalence of resistance, high degree of resistance, the resistance percentages and the resistance patterns of the faecal samples of the pigs and the pig farmers a Chi Square test with continuity correction was performed. A Fisher Exact test was used if the expected frequency in at least one cell was 5 or less. A two-sided significance level of $\leq 0.05$ was used. A Mann-Whitney test was performed to test if significant differences were present between pigs and farmer living at farms with different husbandry methods. A two-sided significance level of $\leq 0.05$ was used.
The error of the method by using the spiral plater and by making tenfold dilutions, calculated from the standard error of the mean, was 0.5 $^{10}$log.

## RESULTS

The data observed for the pig farmers have been presented in a previous study (27). To facilitate comparison with the pig data they are repeated in present study. Ninety-five per cent of the pig farmer colonies and 96% of the pig colonies that grew on Levine-agar, showing the morphology typical of *E. coli*, were identified as *E. coli*. The other colonies tested were also *Enterobacteriaceae*: *Klebsiella* spp., *Citrobacter* spp., *Enterobacter* spp. and *Proteus* spp. In total 291 faecal samples of pigs and 290 samples of humans were received. All of the 291 pig samples showed growth on the agar plates without antibiotics, whereas 12 out of 290 farmer samples failed to grow. So, for the final analysis 291 pig and 278 farmer samples were included. From six pig and 12 farmer samples *E. coli* could not be isolated, because of overgrowth by *Bacillus* spp. or the isolate was indole negative and these were thus excluded from further testing. Finally the antibiotic susceptibility was determined of 285 faecal pig *E. coli* strains and 266 farmer strains, one strain per sample.

*Prevalence of antibiotic resistance*

The prevalences of resistance to neomycin and commonly used drugs in human and pig medicine (i.e. amoxycillin, oxytetracycline, sulphamethoxazole and trimethoprim) ranged from 92-100% in pig faecal samples and from 53%-84% in the farmer samples (Table 1). Significant differences in prevalence of resistance were observed for these antibiotics and for nitrofurantoin. The prevalence of resistance to the other antibiotics tested was distinctly lower in both groups.

*Table 1: Prevalence and high degree of antibiotic resistant Escherichia coli (%) in faecal samples of pigs and pig farmers.*

| Antibiotic | mg/l^ | Prevalence Pigs n=291 | Prevalence Pig Farmers n=278 | High degree Pigs n=291 | High degree Pig Farmers n=278 |
|---|---|---|---|---|---|
| Amx | 25 | 98 | 62* | 11 | 7 |
| Ap** | 32 | 8 | 3 | 0 | 0 |
| Cip | 4 | 2 | 1 | 0 | 0 |
| Flu*** | 4 | 2 | -- | 0 | -- |
| Ft | 50 | 17 | 8 * | 0 | 0 |
| Na*** | 32 | -- | 5 | -- | 1 |
| Ne | 8 | 92 | 66* | 2 | 7 * |
| Ot | 25 | 100 | 79* | 25 | 10* |
| Smx | 100 | 100 | 84* | 24 | 17 |
| Tmp | 8 | 99 | 53* | 7 | 4 |

*mg/l^ = antibiotic concentrations in the agar plates; * = significantly different ($P \leq 0.05$) for a particular antibiotic between pig farmers and pigs; ** = apramycin was only tested for the last 144 pigs and 116 farmers faecal samples; *** = flumequin was only tested for the pig and nalidixic acid only for the human samples; -- = not measured. Amx = amoxycillin, Cip = ciprofloxacin, Flu = flumequin, Na = nalidixic acid, Ne = neomycin, Ft = nitrofurantoin, Ot = oxytetracycline, Smx = sulphamethoxazole, Tmp = trimethoprim, Ap = apramycin.*

### *Degree of antibiotic resistance*

For all frequently used antibiotics a high degree of resistance was observed (Table 1). Significant differences were only observed for neomycin and oxytetracycline.

### *Antibiotic susceptibility of the E. coli isolates*

Most (74%) of the strains isolated from pigs showed resistance to two or more antibiotics, whereas almost half of the farmer isolates (43%) were multi resistant (Figure 1). The percentage of strains showing no resistance to any antibiotic tested was twice as high in the farmers (34%) as in the pigs (15%). Significantly higher resistance percentages to chloramphenicol, nitrofurantoin, oxytetracycline, streptomycin and sulphamethoxazole were observed for the pig isolates. The *E. coli* strains isolated from both the pig farmers and their pigs, that appeared to be nalidixic acid resistant (n=5 and n=1, respectively) were also resistant to flumequin, but susceptible to ciprofloxacin. Moreover, higher $MIC_{90}$ values for trimethoprim and $MIC_{50}$ values for oxytetracycline and streptomycin were observed for the pig isolates (Table 2).

The *E. coli* isolates showed 70 different resistance patterns for the pigs and 52 for the

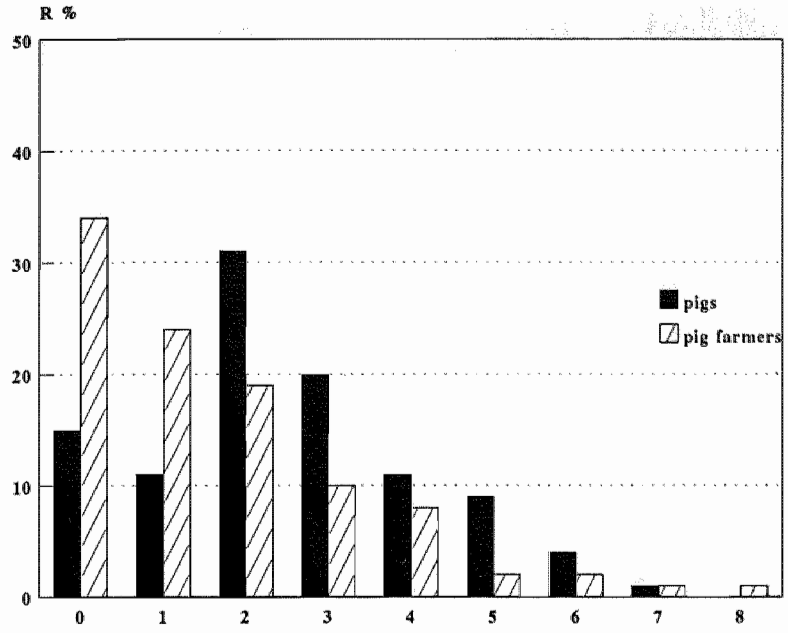


*Figure 1: Antibiotic resistance (%) of E. coli to multiple antimicrobial agents, isolated from faecal pig samples (n=285) and pig farmer samples (n=266), 0 to 8= number of antibiotics, %R= percentage of resistant E. coli isolates.*

farmers. Forty-six and 36 resistance patterns of the pigs and the pig farmers respectively were observed only once or twice. The percentages of the most frequently isolated resistance patterns in pigs and pig farmers differed significantly for: oxytetracycline-streptomycin, oxytetracycline-streptomycin-sulphamethoxazole, amoxycillin and sulphamethoxazole (Table 3). When paired *E. coli* isolates (n=259) of farmers and pigs living at the same farm were compared 10 (4%) of these isolates were resistant to the same antimicrobial agents. The patterns of the remaining 249 combinations showed differences in one, two or more antibiotics (22%, 32% and 38% respectively). These differences were mainly due to differences in resistance to oxytetracycline, streptomycin or sulphamethoxazole.

Recent antibiotic use in pigs (mass-medication) was recorded by 25 out of 291 farms (9%) and 15 farmers out of 290 (5%) and 17 family members (6%) had used antibiotics themselves. Two out of 284 farmers recorded recent hospital stay. From 253 farms we were informed about the type of pig farming. Eighty-seven farms housed only fattening pigs, whereas the other farms (n=166) housed both breeding and fattening pigs.

*Table 2: Antibiotic resistance (R%) and MIC50 and MIC90 of Escherichia coli strains isolated from faecal samples of pigs and pig farmers.*

| Antibiotic | | Pigs (n=285) | | | Pig Farmers (n=266) | | |
|---|---|---|---|---|---|---|---|
| | mg/l^ | MIC50 | MIC90 | R% | MIC50 | MIC90 | R% |
| Amx | 16 | 8 | ≥64 | 25 | 16 | ≥64 | 28 |
| Ap | 16 | 4 | 8 | 0 | 4 | 8 | 0 |
| Amc | 16 | 4 | 16 | 0 | 4 | 8 | 0 |
| C | 8 | 8 | 16 | 13 | 8 | 8 | 7 * |
| Ft | 32 | 16 | 32 | 8 | 16 | 32 | 3 * |
| Na | 8 | 4 | 8 | 0 | 4 | 8 | 2 |
| Ne | 16 | 2 | 4 | 7 | ≤1 | 2 | 3 |
| Ot | 16 | ≥256 | ≥256 | 57 | 8 | ≥256 | 32* |
| S | 16 | 32 | ≥128 | 71 | 4 | ≥128 | 34* |
| Smx | 128 | 64 | ≥1024 | 45 | 32 | ≥1024 | 35* |
| Tmp | 2 | 0.5 | 512 | 16 | 0.5 | 2 | 10 |

*mg/l^= Breakpoint concentration, according to the guidelines of the Dutch Working Party on Susceptibility Testing of Antibiotics (14), except for apramycin which accorded to Hunter (12); *= significantly different resistance percentage R% (P≤0,05) for a particular antibiotic between pig farmers and pigs. MIC50/90= Minimal Inhibitory Concentration (mg/l) at which 50%/90% of the strains are inhibited, R%= resistance percentage. Amc= amoxycillin+clavulanic acid, C= chloramphenicol, S= streptomycin, see also legend Table 1.*

*Table 3: Most frequently isolated patterns of drug resistance in Escherichia coli strains of pigs and/or pig farmers*

| Pattern | Pigs (n=285) n (%) | Pig Farmers (n=266) n(%) |
|---|---|---|
| Ot S | 42 (15) | 10 (4)* |
| Ot S Smx | 27 (10) | 10 (4)* |
| S Smx | 21 (7) | 14 (5) |
| S | 15 (5) | 4 (2) |
| Amx | 2 (1) | 24 (9)* |
| Smx | 1 (0) | 19 (7)* |
| Ot | 6 (2) | 11 (4) |

**= significantly different (P≤0,05) resistance patterns (%) between pig farmers and pigs. Amx= amoxycillin, Ot= oxytetracycline, S= streptomycin, Smx= sulphamethoxazole, Tmp= trimethoprim.*

No significantly different prevalence of resistance could be observed between farmers living at fattening or at mixed farms, except for sulphamethoxazole which showed a higher mean rank for people living at mixed farms. In contrast, pigs living at fattening farms showed significantly higher mean ranks for amoxycillin, neomycin, oxytetracycline and trimethoprim (data not shown).

## DISCUSSION

In the present study faecal pig samples showed significantly higher prevalences and high degrees of resistance to commonly used antibiotics and neomycin than those of the pig farmers. On a yearly basis the total amount of antimicrobials used in The Netherlands in pig medicine is higher (125 mg/kg pig) than in human medicine (75 mg/kg human) (2). The major use of antibiotics in pigs is given orally for mass-medication (100 mg/kg pig), mainly oxytetracycline. As by this medication a total group of pigs is treated with an antibiotic the chance of development and spread of resistance in a herd is higher than after therapy of individual animals. The high use of antibiotics in pig medicine in general and the intensive faecal-oral contact between pigs might explain the high resistance percentages observed in the pigs in this study.

Also differences in antibiotic susceptibility and resistance patterns were observed for pig farmers and pig strains. In faecal samples collected at the same farm only in 4% the same resistance pattern was found. No significant differences could be observed between pigs or people recently treated with antibiotics compared with those not having been treated recently. Therefore, recent antibiotic therapy could not explain the observed differences between pigs and pig farmers. Differences in type of farming were reflected in the group of pigs, suggesting that the farmers form a more uniform group than the pigs. This finding strengthened the idea that the pigs in this study are not the most important source of antibiotic resistant bacteria in the faecal flora of the pig farmers. If transfer of resistant bacteria from pigs to farmers and colonization of the faecal flora of farmers had occurred also differences between the two groups of farmers, working at different farms, might have been expected.

As observed in a previous study (27) pig farmers showed significantly higher resistance percentages than the control group (=(sub)urban residents). A possible explanation could be direct contact with antibiotics e.g. in medicated pig feed. As allergy to veterinary antibiotics such as tylosin (5) is not uncommon in pig farmers, this route cannot be excluded.

Medicated pig feed may contain 0.4-1 g (oxy)tetracycline per kg. A pig farmer might ingest this feed involuntary via hands or inhaled dust. Several studies have shown that small children playing outdoors do not ingest more than 300 mg soil daily (4,6,7). Moreover, during daily activities pig farmers spend approximately five hrs a day in the pig stables. The air in these contains about 6 mg dust /$m^3$ (29). The daily inhalation of dust, for an important part consisting of pig feed, can be estimated at 36 g. Therefore, in the worst case the involuntary inhalation and ingestion of pig feed by pig farmers is about 36 g a day and thus in the extreme case an uptake of 36 mg of (oxy)tetracycline. Moreover, pigs are fed oxytetracycline medicated pig feed less than once a year (2). The defined daily dosage of tetracycline for adult humans is 1000 mg (15). Therefore, any important influence of ingestion of medicated pig feed on the resistance in the faecal flora of pig farmers seems to be very unlikely.

There was no clear explanation for the higher percentage of high degree of resistance to neomycin in the farmer samples. In humans neomycin is used mainly locally and only seldomly orally, whereas in pig medicine the drug is used extensively, especially for the treatment of diarrhoea in young pigs.

Several studies suggested that the use of nalidixic acid selects for resistance to nalidixic acid and nalidixic acid derivatives i.e. the quinolones (9,36). Moreover, it was speculated that especially the veterinary use of fluoroquinolones might lead to an increase in bacterial resistance in human pathogens against this group of antibiotics (13). However, both farmer and pig isolates in this study showed only a low percentage of resistance to nalidixic acid (Table 2). These strains were also resistant to flumequin, but susceptible to ciprofloxacin. This data suggest that nalidixic acid might select for flumequin resistance, but not for ciprofloxacin resistance but, because of the low number of nalidixic acid resistant strains no definite conclusions can be made.

Resistance to chloramphenicol in the pig flora was 13% despite the fact that this drug has not been used for many years in food animals in The Netherlands. It has been officially forbidden in pig medicine since 1990. This has also been observed in Denmark where chloramphenicol in pig medicine has been withdrawn for more than 10 years (1). In the present study only 3 out of 38 chloramphenicol resistant pig isolates showed resistance to chloramphenicol alone. Thirty-five strains were also resistant to oxytetracycline, streptomycin or sulphamethoxazole suggesting a plasmid or chromosomal (mar-A) related resistance (8,23) selected by the use of these antibiotics. The supplementation of pig feed with copper and zinc salts may be an additional selection force, as heavy metal resistance genes are often located on resistance plasmids (35). Also other reasons for the presence of chloramphenicol resistance in the absence of selection pressure by this antibiotic might be

possible. Piglets might become colonized with resistant bacteria derived from other pigs (sows) and the stable environment. Also newly introduced pigs can spread strains to on-site pigs. Therefore, resistant bacteria or resistance plasmids might circulate for a long time on farms. Other sources of resistant bacteria might be survival of bacteria after cleaning the stables or introduction of resistance genes by rodents or contaminated pig feed.

The prevalence and high degree of resistance in the faecal flora and the susceptibility and resistance patterns of the isolates clearly showed differences between both groups. Apart from direct contact with pigs and pig faeces pig farmers are exposed to several factors that might influence antibiotic resistance: antibiotics used for pigs in treatment of pigs and involuntary consumption of pig feed (hand contamination or dust) and personal treatment with antibiotics. As observed in a previous study recent antibiotic use by pig farmers was higher than in (sub)urban residents, but seemed to be of no significant influence on antibiotic resistance in pig farmers (27). It can however, not be excluded that in the long term this personal antibiotic consumption might be responsible for higher numbers of resistant bacteria in the faecal flora of farmers. Direct contact with pigs and pig faeces can also not be excluded as a risk factor, but did not seem to be a major factor in this study. No influence of recent antibiotic treatment of pigs could be observed in this study. However, the influence of mass medication cannot be excluded as a risk factor on antibiotic resistance in pig farmers.

Further studies (including plasmid analysis and transfer experiments) are necessary to investigate similarities between bacterial populations and/or plasmids isolated from the pig farmers and their pigs.

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# *IN VITRO* TRANSFER OF ANTIBIOTIC RESISTANCE BETWEEN FAECAL *ESCHERICHIA COLI* STRAINS ISOLATED FROM PIG FARMERS AND PIGS

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SUMMARY

*Faecal Escherichia coli isolated from pig farmers and pigs were analyzed to biotypes, plasmid patterns and transferability of antibiotic resistance. Two groups of isolates, consisting of pairs of pig farmer and pig strains from the same farm with the same (group A) and with different resistance patterns (group B), were investigated.*

*Only one pair of strains of group A and one of group B showed the same biotype for the pig and pig farmer isolate.*

*The whole plasmid patterns and plasmid profiles after restriction analysis of the paired strains of group A showed only minimal similarities.*

*A striking phenomenon was that in group B 10/11 pig isolates transferred resistance and in group A about 50% of the strains. Transfer experiments showed similar transfer frequencies for pig farmer and pig strains of group A and B.*

*The present study does not provide conclusive evidence for the presence of a common pool of resistance plasmids among pig farmers and their pigs.*

## INTRODUCTION

Antibiotics are considered to be one of the most important discoveries in the history of medicine. However, the extended use and maybe misuse of these compounds has resulted in an increase of resistance in bacteria, which are not sensitive any more to the killing or inhibiting effects of antibiotics. This phenomenon is widely observed in human as well as in veterinary medicine (1,10,22). Not only selection and spread of resistant micro-organisms, but especially intra- and inter-species transfer of antibiotic resistance genes via plasmids has caused this problem of antibiotic resistance (8,20,30). By plasmid analysis i.e. whole plasmid and digesting plasmids with restriction endonucleases it is possible to track antibiotic resistance plasmids in the bacterial population (19,24,29).

A common pool of resistant and susceptible micro-organisms shared by animals and humans is suggested in several studies. From this pool humans can pick up resistant bacteria and resistant genes from animals directly or indirectly. Directly via contact with animals (7,25,26) or indirectly by ingesting foods contaminated with faecal flora of food animals (11,15,18). Evidence for the existence of a common pool of micro-organisms or even resistance genes and the transfer of resistance plasmids from animals to humans has been provided by Levy (12,13) and others (14,15,20,21,29). However, O'Brien *et al.* (24) strongly suggested different plasmid populations in poultry and poultry workers. Moreover, Jansson *et al.* (8) demonstrated in only one out of 400 trimethoprim resistant *Enterobacteriaceae* isolated from Swedish individuals the presence of dihydrofolate reductase type IX, a gene which is common in pig isolates in Sweden.

The aim of the present study was to analyze the genetic basis of resistance of *Escherichia coli* strains isolated from the faecal flora of pig farmers and their pigs by plasmid analysis and transferability of antibiotic resistance. The analysis of the antibiotic resistant donor strains and transconjugants included the comparison of biotypes and phenotypes as well as genotypes of the resistance patterns. Besides transfer to a laboratory strain, *E. coli* K12, also matings with two susceptible wild *E. coli* strains were tested, to analyze the influence of the recipient strains used.

## MATERIALS AND METHODS

### Bacterial strains and study population

Donor strains were selected from our collection of *E. coli* isolated from faecal samples of

pig farmers and of pigs on their farms (23). If a common pool of resistance genes should exist, from which transfer of these genes could take place, it was expected that pig farmer and pig isolates collected at the same farm and showing the same resistance pattern could easily transfer their plasmids. In addition it was expected that the plasmid sizes of pig-pig farmer combinations with the same resistance pattern were similar. In contrast, however, pig farmer and pig isolates collected at the same farm, but showing different resistance patterns were expected to show less similarities. To analyze these suggestions two groups of strains were selected and tested. Group A consisted of 10 pairs of isolates of pig farmers and pigs from the same farm, which both had the same resistance pattern. Group B included 13 at random selected pairs of strains isolated from a pig farmer and pigs living at the same farm, but showing different resistance patterns. Either the pig or the pig farmer isolate of group B had a similar resistance pattern as the strains in group A. The matching strain from the same pair of group B had a different resistance pattern.
The nalidixic acid resistant recipient strain *E. coli* K12 was susceptible to all antibiotics to which resistance was tested.
Biotypes of the strains used were determined using API-20E biochemical system (BioMerieux, Den Bosch, The Netherlands) according to the instructions of the manufacturer.

**Plasmid isolation and analysis**

Plasmid DNA was extracted by a modification of the method described by Kado and Liu (9). The molecular weights of the plasmids were determined by agarose gel electrophorese using 0.7% agarose gel and running at 75V during four hours. Ethidiumbromide was used to visualize DNA with ultraviolet light after running was completed. A *Salmonella typhimurium* strain (kindly provided by Dr. N. van Leeuwen, RIVM) containing five plasmids (91, 39, 7.6, 5.8 and 4.4 Kb) was used as reference.
Whole DNA content from donor/transconjugant strains, showing resistance to the most prevalent pattern oxytetracycline-streptomycin-sulphamethoxazole (OtSSmx), was digested with restriction endonuclease EcoR1 according to the instructions of the manufacturer and extracted by an alkaline lysis method (27). Plasmid isolation and restriction analysis was minimal performed twice. Examples are shown in Figures 1, 2 and 3.

**Transfer of resistance**

Conjugation experiments were performed by broth mating as follows: overnight cultures (37°C) in BHI (brain heart infusion bouillon, Oxoid CM225) of recipient and donor strains were 1:10 diluted in BHI and shaken for two hours (37°C). Equal volumes (2 ml) of donor and recipient were put together in 2 ml BHI and incubated for another two hours

at 37°C with gently shaking to allow conjugation. Dilutions ($10^0$-$10^{-5}$) of the conjugation mixture were inoculated on Iso-sensitest$^R$ agar plates (Oxoid CM471) containing nalidixic acid 32 mg/l and one of the antibiotics to which the donor was resistant. If no conjugation was observed after two hours the mating was repeated overnight at 37°C without agitation. Those strains which than still failed to transfer resistance were not further examined.

The frequencies of transfer were calculated as the ratio between the number of transconjugants and the number of colony forming units (CFU) of the donor strain. Depending on the antibiotic used for the selection the frequency of transfer may vary. For sake of simplicity in the Tables 1,2 and 4 the mean frequency of transfer is given.

The antibiotics and concentrations used in the selective plates were : 4 mg/l trimethoprim; 20 mg/l neomycin and streptomycin; 32 mg/l amoxycillin, chloramphenicol and oxytetracycline and 256 mg/l sulphamethoxazole. After overnight incubation at 37°C from each selective plate three *E. coli*-like colonies were picked up randomly and tested for the indole reaction and for growth at 42°C. If the reactions were positive the isolates were considered to be *E. coli* and stored at -20°C until use. As control, the same procedure was followed using donor with BHI as recipient instead of *E. coli* K12 overnight culture and recipient with BHI.

Co-transfer of resistance to other than the antibiotics used for selection was analyzed by susceptibility testing (as described before, 23) of the transconjugants.

For further experiments six pig farmer and six pig strains were selected as donor based on differences in frequency of transferring resistance to *E. coli* K12, after two hours of mating. From both pig and pig farmer isolates three strains with a frequency less than 5 log and three with a frequency of 5 log or more (Table 4) were randomly chosen. Likewise one pig and one human wild strain, susceptible to the antibiotics tested, were selected from our collection of faecal *E. coli* isolated from pigs and healthy residents (17,23) and used as recipient in those mating experiments. The recipient strains were made nalidixic acid resistant by several passages on nalidixic acid (32 mg/l) containing Iso-sensitest$^R$ agar plates.

Finally conjugation experiments were performed between the five susceptible strains of group B with their corresponding resistant strains.

*Table 1: Biotypes, resistance patterns and conjugation, to E. coli K12, frequencies of donor E. coli strains isolated from faecal samples of pigs and pig farmers of group A (both showing the same resistance pattern).*

| Group | nr | API | R pattern | TFm | range |
|---|---|---|---|---|---|
| P | 61 | 1044512 | OtS | ... | |
| | 77 | 5144572 | OtSSmx | $9.0x10^{-9}$ | $(0\text{-}2.7x10^{-8})$ |
| | 80 | 7144572 | OtSSmx | $4.3x10^{-10}$ | $(0\text{-}1.3x10^{-9})$ |
| | 86 | 7044552 | AmxCOtSSmxTmp | $2.3x10^{-4}$ | $(1.6x10^{-4}\text{-}3.1x10^{-4})$ |
| | 121 | 5044572 | SmxTmp | $2.7x10^{-5}$ | $(2.5x10^{-5}\text{-}2.9x10^{-5})$ |
| | 188 | 1004552 | OtSSmx | ... | |
| | 195 | 5044552 | AmxS | ... | |
| | 205 | 5044552 | SSmx | ... | |
| | 210 | 5144542 | AmxOtSSmxTmp | $6.6x10^{-6}$ | $(0\text{-}2.6x10^{-5})$ |
| | 243 | 5144572 | OtS | $5.0x10^{-6}$ | $(0\text{-}1.0x10^{-5})$ |
| PF | 61 | 5144572 | OtS | ... | |
| | 77 | 1144552 | OtSSmx | ... | |
| | 80 | 5144552 | OtSSmx | $3.9x10^{-8}$ | $(0\text{-}3.2x10^{-8})$ |
| | 86 | 5144172 | AmxCOtSSmxTmp | $2.3x10^{-6}$ | $(0\text{-}8.6x10^{-6})$ |
| | 121 | 5144572 | SmxTmp | $3.7x10^{-5}$ | $(3.2x10^{-5}\text{-}4.2x10^{-5})$ |
| | 188 | 5144572 | OtSSmx | $7.7x10^{-5}$ | $(2.7x10^{-8}\text{-}1.9x10^{-4})$ |
| | 195 | 5044572 | AmxS | ... | |
| | 205 | 5044542 | SSmx | ... | |
| | 210 | 5144572 | AmxOtSSmxTmp | $8.6x10^{-5}$ | $(0\text{-}2.1x10^{-4})$ |
| | 243 | 5144572 | OtS | ... | |

*nr= strain number, API= biotype according to API 20-E biochemical system, R pattern= resistance pattern, TFm= mean transfer frequency to E. coli K12, range= range of transfer frequencies of transconjugants selected from several selective plates, ...= no conjugation observed after overnight conjugation. P= pig, PF= pig farmer, Amx= amoxycillin, C= chloramphenicol, Ot= oxytetracycline, S= streptomycin, Smx= sulphamethoxazole, Tmp= trimethoprim.*

## RESULTS

### Biotypes

Only one pair of strains of group A and one of group B showed exactly the same biotype and three pairs of each group were similar (only differing in fermentation of one sugar). The biotypes 5044552 and 5144572 were most prevalent among both groups of strains (Tables 1,2).

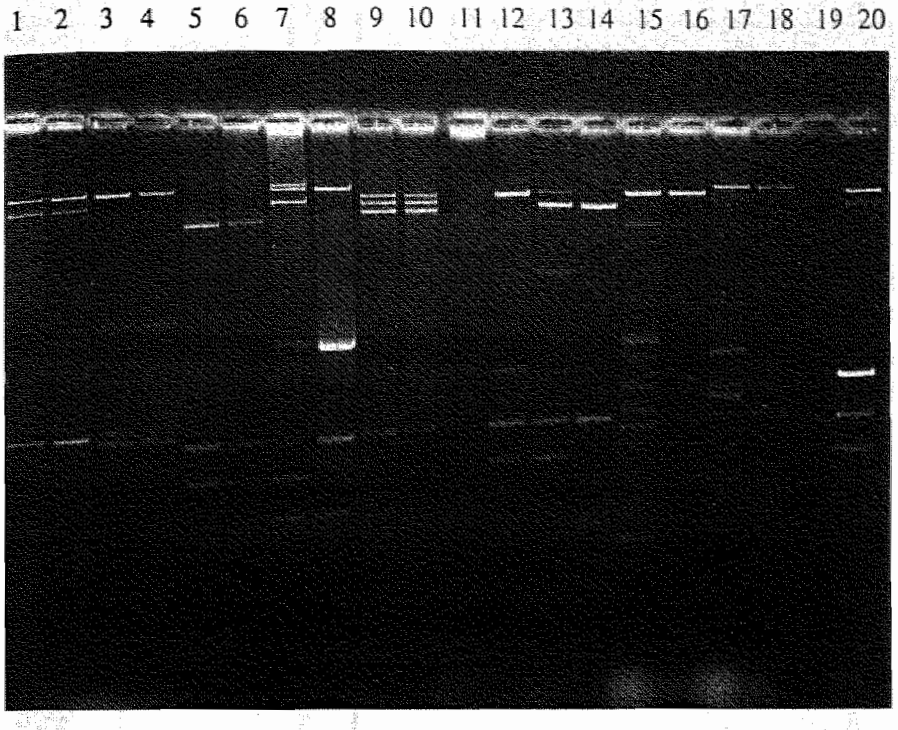


*Figure 1: Agarose gel electrophoresis of plasmid DNA extracted from E. coli donor and transconjugant strains of pig farmers and pigs of group A (lane 1-14) and B (lane 15-18) showing resistance to OtSSmx. Lane 1, 3, 5, 7, 9, 11 and 13 are pig donor strains 42, 123, 128, 155, 181, 260 and 289, respectively. Lane 15, 17 and 18 are pig farmer donor strains 48, 65 and 133, respectively. The even numbers are the transconjugants of the preceding donor strains, except for lane 18. Lane 20 shows the reference Salmonella typhimurium strain with size standards in descending order 91, 39, 7.6, 5.8 and 4.4 Kb.*

**Plasmid patterns of pig (P) and pig farmer (PF) strains**

The plasmid pattern of the pig and pig farmer strains used are shown in Table 3, no plasmids were detectable in two strains (PF155 and PF260) with phenotypes sulphamethoxazole and amoxycillin-sulphamethoxazole, respectively. The number of plasmids ranged from one to 11, plasmid sizes from 204 Kb to 1.4 Kb. Both groups showed diverse whole plasmid patterns per resistance phenotype. Comparing the plasmid profiles of strains with resistance pattern OtSSmx no obvious similarities between pig and pig farmer isolates could be observed for group B strains (Figure 1). Even group A isolates from pigs and pig farmers living at the same farm showed different plasmid patterns (Figure 2). Further analysis of the OtSSmx resistant isolates using EcoR1 digestion of plasmid DNA showed distinct different profiles (Figure 3).

*Table 2: Biotypes, resistance patterns and conjugation, to E. coli K12, frequencies of donor E. coli strains isolated from faecal samples of pigs and pig farmers of group B (both showing different resistance patterns).*

| Group | nr | API | R pattern | TFm | range |
|---|---|---|---|---|---|
| P | 15 | 5144552 | SmxTmp | $1.4x10^{-5}$ | ($1.2x1^{-7}$-$2.7x10^{-5}$) |
| | 42 | 5144572 | OtSSmx | $2.4x10^{-5}$ | ($1.6x10^{-5}$-$2.9x10^{-5}$) |
| | 48 | 5044552 | OtS | ... | |
| | 65 | 5144552 | ** | | |
| | 85 | 5044542 | NeOtSSmx | $1.9x10^{-5}$ | ($0$-$4.8x10^{-5}$) |
| | 123 | 5144552 | OtSSmx | $1.3x10^{-8}$ | ($0$-$4.0x10^{-8}$) |
| | 128 | 5144572 | OtSSmx | $4.6x10^{-6}$ | ($1.2x10^{-7}$-$4.3x10^{-6}$) |
| | 133 | 5144572 | ** | | |
| | 155 | 5044552 | OtSSmx | $6.9x10^{-6}$ | ($2.8x10^{-6}$-$1.1x10^{-5}$) |
| | 161 | 5444542 | SmxTmp | $3.9x10^{-6}$ | ($4.2x10^{-7}$-$3.5x10^{-6}$) |
| | 181 | 5044552 | OtSSmx | $1.9x10^{-7}$ | ($0$-$6.4x10^{-7}$) |
| | 260 | 5444552 | OtSSmx | $2.2x10^{-7}$ | ($0$-$6.5x10^{-7}$) |
| | 289 | 5144552 | OtSSmx | $8.8x10^{-8}$ | ($0$-$2.2x10^{-7}$) |
| PF | 15 | 5044552 | NeOtSSmxTmp | $2.9x10^{-5}$ | ($0$-$8.3x10^{-5}$) |
| | 42 | 5044552 | SSmx | $4.7x10^{-8}$ | ($0$-$9.4x10^{-8}$) |
| | 48 | 5044552 | OtSSmx | $7.6x10^{-5}$ | ($0$-$1.4x10^{-4}$) |
| | 65 | 5044172 | OtSSmx | ... | |
| | 85 | 7144572 | SSmx | ... | |
| | 123 | 5144572 | ** | | |
| | 128 | 5144552 | ** | | |
| | 133 | 5044572 | OtSSmx | ... | |
| | 155 | 5044552 | Smx | ... | |
| | 161 | 5044552 | ** | | |
| | 181 | 5044552 | AmxCNeOtS | ... | |
| | 260 | 5144572 | AmxSmx | ... | |
| | 289 | 5144572 | AmxOtS | $1.1x10^{-6}$ | ($0$-$4.5x10^{-6}$) |

*Legend see Table 1, ** = not resistant, Ne = neomycin.*

**Transfer of resistance**

In group A about 50% of the strains tested, six out of ten pig and five out of ten pig farmer strains, were able to transfer antibiotic resistance to *E. coli* K12 (Table 1). For the group B isolates the figures were ten out of 11 pig and four out of ten resistant pig farmer isolates (Table 2).

In group A plasmid transfer from antibiotic resistant *E. coli* strains, isolated from pig farmer and pigs from the same farm, to *E. coli* K12 was observed in four out of the ten

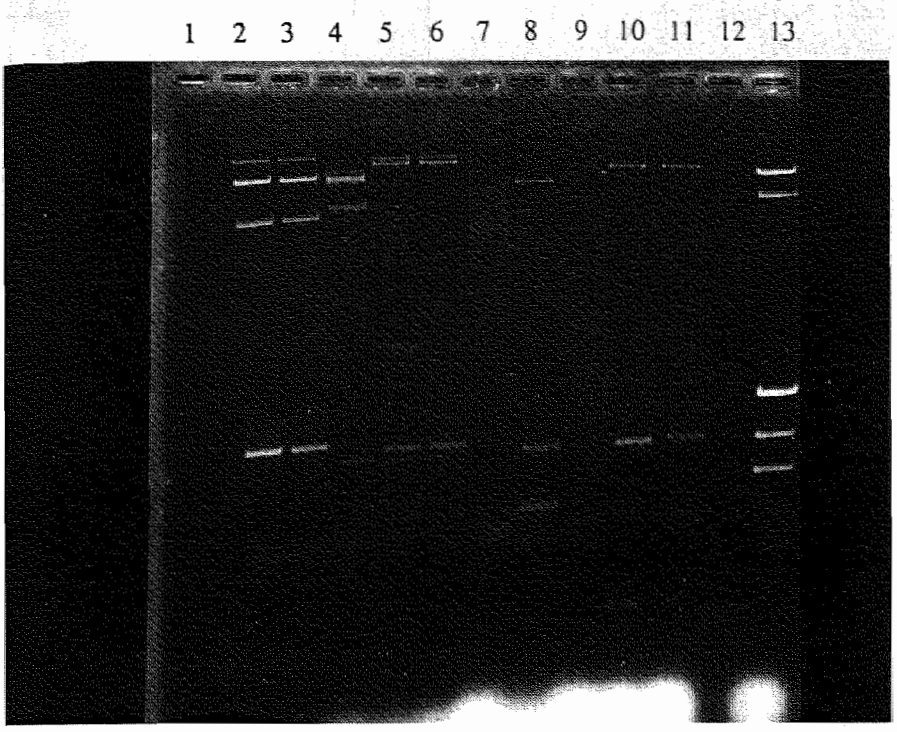


*Figure 2: Agarose gel electrophoresis of plasmid DNA extracted from E. coli donor (D) and transconjugant (T) strains of pig farmers and pigs of group A showing resistance to OtSSmx. Lane 2= D P77, lane 3= T P77, lane 4= D PF77, lane 5= D P80, lane 6= T P80, lane 7= D PF80, lane 8= T PF80, lane 9= D P188, lane 10= D PF188, lane 11= T PF188 and lane 13 shows the reference strain. Footnote: unfortunately no visible plasmids could be observed for donor strains PF80 and P188 on this gel in lane 7 and 9, respectively. Previous plasmid isolations did show the presence of plasmids in these strains (Table 3).*

combinations (Table 1). With three other pairs no transfer was observed. In another two pairs (nr. 77 and 243) only the pig strains transferred their resistance and of pair 188 only the pig farmer isolate showed transferable resistance. The mean frequency of transfer of the group A pig isolates ranged from $4.3 \times 10^{-10}$ to $2.3 \times 10^{-4}$ and for the pig farmer strains from $3.9 \times 10^{-8}$ to $8.6 \times 10^{-5}$.

In group B two pig isolates (nr. 65 and 133) were susceptible, whereas the corresponding pig farmers isolates, both resistant to OtSSmx, did not transfer their resistance to *E. coli* K12 (Table 2). In contrast, three pig isolates (nr. 123, 128 and 161) did show transferable resistance of OtSSmx to *E. coli* K12, whereas the corresponding pig farmer strains were susceptible. Of three other pairs (nr. 155, 181 and 260) both pig farmer and pig isolates

*Table 3: Plasmid profiles (sizes in Kb) and resistance patterns of donors and transconjugants after conjugation with E. coli K12.*

| | Donor | | Transconjugant | |
|---|---|---|---|---|
| nr | R pattern | Plasmid profile | R transferred | Plasmid(s) transferred |
| **Group A** | | | | |
| PF210 | AmxOtSSmxTmp | 107 91 19 5.8 4.4 1.8 | AmxOt | 107 91 |
| | | | AmxOt Smx | 107 |
| | | | AmxOt SmxTmp | 107 91 |
| | | | SSmxTmp | 91 4.4 1.8 |
| P 210 | AmxOtSSmxTmp | 56 19 5.8 | AmxOtSSmxTmp | 56 19 5.8 |
| PF 86 | AmxCOtSSmxTmp | 81 45 3.9 3.2 | AmxCOtSSmxTmp | 81 |
| | | | AmxCOtSSmxTmp | 81 45 7.6 5.8 3.9 3.2 |
| | | | AmxCOtSSmxTmp | 81 45 |
| P 86 | AmxCOtSSmxTmp | 107 81 2.6 | AmxCOtSSmxTmp | 107 81 |
| PF121 | SmxTmp | 112 5.8 2.7 | SmxTmp | 74 6.0 2.7 |
| P 121 | SmxTmp | 74 6.0 2.7 | SmxTmp | 74 6.0 2.7 |
| PF205 | SSmx | 5.8 | ... | |
| P 205 | SSmx | 91 6.8 5.8 3.5 | ... | |
| PF195 | AmxS | 107 39 17 | ... | |
| P 195 | AmxS | 107 91 39 | ... | |
| PF 61 | OtS | 91 76 28 10 5.1 3.5 | ... | |
| P 61 | OtS | 91 76 3.9 | ... | |
| PF243 | OtS | 135 91 3.9 | ... | |
| P 243 | OtS | 105 56 | OtS | 105 |
| PF 77 | OtSSmx | 79 59 28 4.6 1.4 | ... | |
| P 77 | OtSSmx | 110 52 24 11 5.6 4.8 | OtSSmx | 110 52 24 11 5.6 4.8 |
| PF 80 | OtSSmx | 59 5.1 3.1 | OtSSmx | 59 5.1 3.1 |
| P 80 | OtSSmx | 135 110 11 10 5.1 4.6 3.1 | OtSSmx | 135 110 11 10 5.1 3.1 |
| PF188 | OtSSmx | 204 100 5.4 1.7 | OtSSmx | 204 100 5.4 1.7 |
| | | | SSmx | 100 |
| P 188 | OtSSmx | 107 39 5.8 2.6 | ... | |

*Table 3: continued*

| | Donor | | Transconjugant | |
|---|---|---|---|---|
| nr | R pattern | Plasmid profile | R transferred | Plasmid(s) transferred |
| **Group B** | | | | |
| P 42 | OtSSmx | 36 22 4.4 | OtSSmx | 36 22 8.9 4.4 |
| PF 48 | OtSSmx | 65 22 9.3 8.5 7.8 6.9 6.0 5.2 4.7 2.3 | SSmx | 65 |
| PF 65 | OtSSmx | 102 12 8.9 6.9 6.5 5.6 4.9 3.6 3.2 2.4 1.9 | ... | |
| P 123 | OtSSmx | 49 13 4.8 4.4 2.2 | OtSSmx | 49 13 4.8 4.4 2.2 |
| P 128 | OtSSmx | 21 4.3 3.2 | OtSSmx | 21 4.3 3.2 |
| | | | SSmx | 21 4.3 3.2 |
| PF133 | OtSSmx | 102 5.8 | ... | |
| P 155 | OtSSmx | 102 78 35 9.1 4.6 3.5 2.6 | OtSSmx | 78 9.1 4.6 2.6 |
| P 181 | OtSSmx | 49 30 23 4.8 | OtSSmx | 49 30 23 4.8 |
| P 260 | OtSSmx | 56 7.8 5.2 4.1 | OtSSmx | 56 7.8 5.2 4.1 |
| P 289 | OtSSmx | 65 35 15 5.2 4.1 | Ot | 65 35 |
| | | | SSmx | 35 5.4 |
| P 48 | OtS | 14 | ... | |
| P 15 | SmxTmp | 74 22 6.0 | SmxTmp | 74 6.0 |
| P 161 | SmxTmp | 74 6.0 1.8 | SmxTmp | 28 7.8 |
| | | | SmxTmp | 74 6.0 |
| PF 42 | SSmx | 162 14 13 8.1 7.1 4.4 | SSmx | 162 14 8.1 7.1 5.8 4.4 |
| PF 85 | SSmx | 18 8.9 7.1 5.8 4.8 2.9 2.6 1.9 | ... | |
| PF289 | AmxOtS | 91 39 6.5 5.8 | OtS | 91 39 |
| PF181 | AmxCNeOtS | 30 9.5 7.6 6.8 | ... | |
| P 85 | NeOtSSmx | 112 6.2 5.8 | NeOtSSmx | 112 |
| PF 15 | NeOtSSmxTmp | 55 28 6.5 4.9 | NeOtSSmxTmp | 155 55 6.5 |
| | | | NeOtSSmxTmp | 55 6.5 |
| PF260 | AmxSmx | ** | | |
| PF155 | Smx | ** | | |

*Legend see Table 1.*

*Table 4: Resistance patterns and conjugation frequencies of donor E. coli strains isolated from faecal samples of pigs, pig farmers and (sub)urban residents, conjugated with three E. coli recipients of different origin.*

| nr | R pattern | recipient | TFm | range |
|---|---|---|---|---|
| P 15 | SmxTmp | K12 | $1.4x10^{-5}$ | $(1.2x10^{-7}$-$2.7x10^{-5})$ |
| | | Pig | $5.2x10^{-5}$ | $(5.0x10^{-5}$-$5.3x10^{-5})$ |
| | | Human | $4.7x10^{-5}$ | $(9.2x10^{-8}$-$4.7x10^{-5})$ |
| P 42 | OtSSmx | K12 | $2.4x10^{-5}$ | $(1.6x10^{-5}$-$2.9x10^{-5})$ |
| | | Pig | $1.2x10^{-5}$ | $(3.5x10^{-6}$-$2.7x10^{-5})$ |
| | | Human | $4.2x10^{-5}$ | $(0$-$1.0x10^{-4})$ |
| P 86 | AmxCOtSSmxTmp | K12 | $2.3x10^{-4}$ | $(1.6x10^{-4}$-$3.1x10^{-4})$ |
| | | Pig | $3.9x10^{-6}$ | $(2.3x10^{-6}$-$5.0x10^{-6})$ |
| | | Human | $5.5x10^{-6}$ | $(3.6x10^{-7}$-$1.2x10^{-5})$ |
| P121 | SmxTmp | K12 | $2.7x10^{-5}$ | $(2.5x10^{-5}$-$2.9x10^{-5})$ |
| | | Pig | $4.4x10^{-5}$ | $(4.0x10^{-5}$-$4.8x10^{-5})$ |
| | | Human | $2.9x10^{-5}$ | $(1.3x10^{-5}$-$1.6x10^{-5})$ |
| P128 | OtSSmx | K12 | $4.6x10^{-6}$ | $(1.2x10^{-7}$-$4.3x10^{-6})$ |
| | | Pig | $1.2x10^{-7}$ | $(0$-$3.6x10^{-7})$ |
| | | Human | $1.4x10^{-6}$ | $(0$-$4.2x10^{-6})$ |
| P155 | OtSSmx | K12 | $6.9x10^{-6}$ | $(2.8x10^{-6}$-$1.1x10^{-5})$ |
| | | Pig | $1.8x10^{-4}$ | $(1.5x10^{-4}$-$2.4x10^{-4})$ |
| | | Human | $2.8x10^{-6}$ | $(7.0x10^{-9}$-$2.8x10^{-6})$ |
| PF48 | OtSSmx | K12 | $7.6x10^{-5}$ | $(0$-$1.4x10^{-4})$ |
| | | Pig | $3.2x10^{-7}$ | $(3.5x10^{-8}$-$2.5x10^{-7})$ |
| | | Human | $5.3x10^{-10}$ | $(0$-$1.6x10^{-9})$ |
| PF80 | OtSSmx | K12 | $3.9x10^{-8}$ | $(0$-$3.2x10^{-8})$ |
| | | Pig | $6.7x10^{-9}$ | $(0$-$2.0x10^{-8})$ |
| | | Human | $5.3x10^{-9}$ | $(0$-$1.2x10^{-8})$ |
| PF86 | AmxCOtSSmxTmp | K12 | $2.3x10^{-6}$ | $(0$-$8.6x10^{-6})$ |
| | | Pig | $3.7x10^{-8}$ | $(0$-$1.1x10^{-7})$ |
| | | Human | $6.4x10^{-5}$ | $(2.1x10^{-9}$-$1.4x10^{-4})$ |
| PF121 | SmxTmp | K12 | $3.7x10^{-5}$ | $(3.2x10^{-5}$-$4.2x10^{-5})$ |
| | | Pig | $1.2x10^{-4}$ | $(1.0x10^{-4}$-$1.4x10^{-4})$ |
| | | Human | $3.7x10^{-4}$ | $(3.3x10^{-4}$-$4.1x10^{-4})$ |
| PF188 | OtSSmx | K12 | $7.7x10^{-5}$ | $(2.7x10^{-8}$-$1.9x10^{-4})$ |
| | | Pig | $3.4x10^{-6}$ | $(0$-$6.0x10^{-6})$ |
| | | Human | $2.4x10^{-9}$ | $(1.8x10^{-9}$-$3.6x10^{-9})$ |
| PF210 | AmxOtSSmxTmp | K12 | $8.6x10^{-5}$ | $(0$-$2.1x10^{-4})$ |
| | | Pig | $4.2x10^{-5}$ | $(0$-$1.5x10^{-5})$ |
| | | Human | $3.8x10^{-6}$ | $(0$-$1.9x10^{-5})$ |

*K12= recipient strain E. coli K12, Pig= porcine E. coli recipient strain, Human= human E. coli recipient strain, see also legend Table 1.*

were resistant to different antibiotics, only the pig isolates showed transferable resistance. The group B isolates transferred their resistance with mean frequencies ranging from $1.3x10^{-8}$ to $2.4x10^{-5}$ for the pig strains and from $4.7x10^{-8}$ to $7.6x10^{-5}$ for the pig farmers isolates (Table 2).

Moreover, no transconjugants could be isolated after mating resistant pig strains (n=2) of group B with corresponding susceptible pig farmer strains. Similarly, from the same group resistant pig farmer (n=3) isolates did not transfer their resistance to corresponding susceptible pig isolates.

**Resistance patterns of transconjugants** (Table 3)

The resistance phenotypes of the transconjugants of both pig and pig farmer isolates were in general similar to those of the donor strains. Although selection on streptomycin or oxytetracycline containing agar plates yielded not always transconjugants, selection on other antibiotics (e.g. sulphamethoxazole or amoxycillin) did result in general in transconjugants with the same resistance pattern as those of the donor isolates. Exceptions were found for P289, PF48 and PF210 which transferred only part of their resistance to *E. coli* K12, whereas transconjugants from P128 and PF188 showed either a similar resistance pattern as that of the donor or were resistant to streptomycin-sulphamethoxazole only.

**Transfer of resistance to different recipients**

The frequencies of transfer from the antibiotic resistant pig isolates either to *E. coli* K12, or to susceptible pig or human recipient strains, were quite similar (Table 4). Only the human strain was a less efficient recipient for the donor isolates PF48 and PF188 than *E. coli* K12. In contrast transfer from PF86 to the human recipient tended to occur with a higher frequency than to the pig recipient. Although not all plasmids observed in the donor strains could be detected in their transconjugants the plasmid patterns obtained with the different recipients were in general similar to those of the donor strain.

## DISCUSSION

Several studies strongly suggest the presence of a common pool of resistance in micro-organisms endogenous to the intestinal flora of humans and animals, from which transfer of resistance to bacteria belonging to the flora of other animals and humans may take

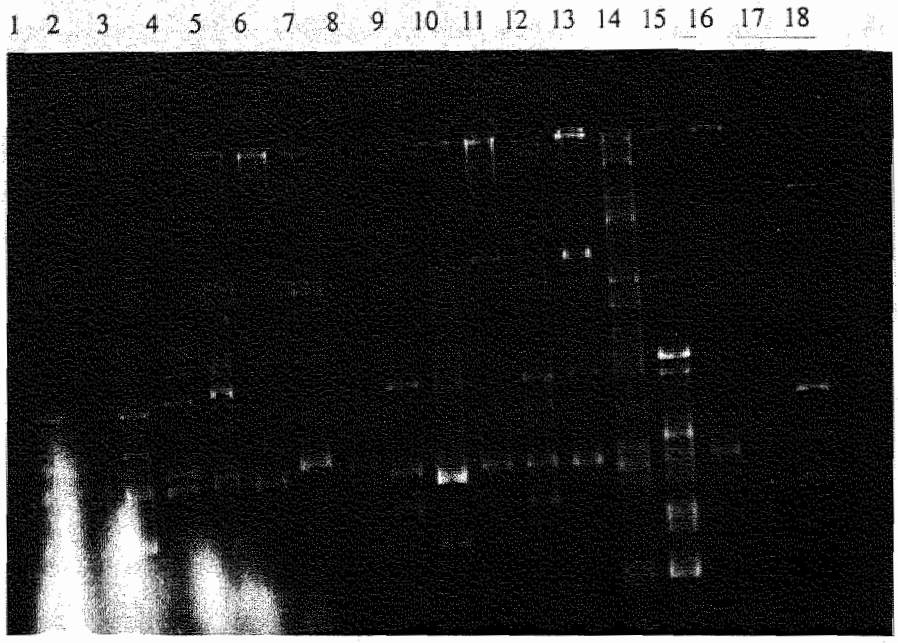


*Figure 3: Agarose gel electrophoresis of plasmid DNA fragments from EcoR1 digests from OtSSmx resistant transconjugant (T) and donor (D) E. coli isolates of group A and B. Lane 1= T P77, lane 2= T P80, lane 3= T PF80, lane 4= T PF188, lane 5= D PF77, lane 6= D P188, lane 7= T P42, lane 8= T P123, lane 9= T P128, lane 10= T P155, lane 11= T P181, lane 12= T P260, lane 13= T P289, lane 14= T PF48, lane 15= D PF65, lane 16= D PF133 and lane 18 shows the reference strain.*

place (12,14,15). However, this hypothesis is not confirmed by others (24). In an attempt to provide some evidence for this hypothesis we analyzed biotypes, plasmids, plasmidprofiles and transfer frequencies of plasmids of strains isolated from faecal samples of pig farmers and their pigs. In this study two groups of strains isolated from the faecal flora of pig farmers and pigs living at the same farm were analyzed. Group A included pairs of pig farmer and pig isolates with the same resistant patterns and group B included pairs of strains with different resistance patterns, but one of the isolates of each pair was the same as the resistance patterns observed in group A. If a common pool of bacteria or plasmids should exist, which easily could be transferred, it is to be expected that plasmid patterns of pig-pig farmer combinations of group A isolates should show more similarities than the patterns of group B isolates. In addition it might be expected that strains from group A could transfer their resistance more easily than the group B isolates.

In the literature the ability of multi resistant *E. coli* strains to transfer resistance to *E. coli*

K12 ranged from 26% to 61% for human isolates and from 50% to 76% for pig derived strains (1,2,3,4,5,6,16,19,21,24). The variation in transferability between the different studies can be explained by differences in antibiotic concentration used in the selective plates and/or by differences in methods used. However, overall there is a tendency for a higher transferability for pig isolates compared to human strains. Similar observations were done in the present study (Tables 1,2). Although differences in ability to transfer resistance were observed, the transfer frequencies of pig and pig farmer strains were in the same size of order and ranged between $10^{-10}$ and $10^{-4}$ and between $10^{-8}$ and $10^{-5}$, respectively. The transferability to *E. coli* K12 of the pig and pig farmer strains of group A was similar, but of group B more pig isolates (ten out of 11) showed transfer of resistance than the pig farmer strains (four out of ten).

Combinations of OtSSmx were frequently observed resistance patterns in the original group of pigs examined (23). Also in other countries this transferable resistance pattern has been observed frequently in pig populations (1,28). Smith (28) suggested that these pigs constitute a reservoir of transferable resistance genes and therefore pigs might be a possible source of resistance for human intestinal bacteria. In the present study six strains (three pairs) of group A and ten strains of group B (three PF and seven P strains) showed resistance to OtSSmx. In group A OtSSmx resistance was transferable in two PF and two P isolates, however, transfer of OtSSmx by both pig and pig farmer strains was observed in pair 80 only. The plasmid patterns of these donor strains showed only two plasmids in common (5.1 and 3.1 Kb). Pig farmer and pig donor strains of pair 77 and pair 188 of group A shared no plasmids. In group B in one PF and all P isolates OtSSmx resistance were transferable. The highest frequencies of transfer were observed for two pig farmer strains (nr. 48 and 188). More information about the homogeneity of the OtSSmx resistant isolates was obtained by restriction endonuclease analysis. After EcoR1 digestion of the plasmid DNA of those strains more than half of the bands obtained were different. These findings suggest the absence of a relationship between these isolates (24). In general the present study showed only minimal similarity between whole plasmid patterns in pig and pig farmer strains with the same resistance phenotype.

Most pairs of strains of Group A and B showed different biotypes, not corresponding with the antibiotic resistance patterns. As biotypes are in general encoded by chromosomally based genes and antibiotic resistance is mainly extrachromosomally on plasmids (21) lack of relationships between both, as observed in the present study, could have been expected. As the observed biotypes of corresponding strains differed it was suggested that the dominant intestinal *E. coli* flora of pig farmer and pig living at the same farm were

not the same. In the present study mainly differences, as to biotypes and antibiotic resistance phenotypes, between pig and pig farmer strains were observed. Although the transferability of plasmids of pig strains was higher than of pig farmer strains, matings with recipient strains of different origins (the laboratory strain *E. coli* K12 and the wild pig and human strains) showed similar transfer frequencies, suggesting the possibility of transfer of resistance plasmids in both directions is similar. However, the reciprocal conjugation experiments of resistant pig farmer strains of group B with the susceptible wild strain isolated from pigs of the corresponding pig farmer, and vice versa, showed no transfer of plasmids. Although only five strains were investigated, the *in vitro* results do not support a readily *in vivo* transfer of resistance plasmids between pig and pig farmer strains. Whether the *in vitro* results are applicable for *in vivo* transfer of plasmids from animals to man is not yet known. Some studies mentioned *in vivo* transfer of resistance plasmids from chicken to man, whereas others did not (12,15,24). Spread of a resistance plasmid between chickens of the same cage and transfer of this plasmid from chicken to man have been described by Levy *et al.* (12). In two out of fourteen persons the presence of strains containing that particular plasmid was demonstrated in their faecal samples. During the two month study period the samples were only once positive. An explanation for this short period of colonization is difficult to give, but may be the selective pressure by tetracycline in the chicken feed and the absence of this selective pressure in humans. Linton *et al.* (15) described in one out of five volunteers *in vivo* transfer of strains from chicken to man after handling and preparing chicken carcasses without eating them. Restriction enzyme analysis showed similarity of the isolates. Also in this study the colonization of the human gut with the chicken strain was only for a short period of time i.e. ten days, but this does not exclude the possibility of transfer of resistance plasmids. Especially in persons taking antibiotics this might occur at a much lower inoculum level. In a recent study of O'Brien *et al.* (24) at a poultry processing plant no similarity was demonstrated between poultry *E. coli* and *E. coli* isolated from women working at the poultry processing line, none of the plasmids from the human isolates was similar to those from chicken strains. Our findings were in line with these data, only minimal similarities were observed between pairs of isolates of group A concerning biotypes and plasmid patterns.

As the transferability of the pig strains of group A was lower than of group B isolates and the transfer frequencies were of the same size of order the presence of a common pool of bacterial strains or plasmids between the farmers and pigs seemed unlikely. This is supported by the results of the EcoR1 digestion of OtSSmx resistant strains.

We conclude, that the data of the present study did not provide conclusive evidence for

the existence of a common pool of resistance plasmids among pig farmers and their pigs.

## ACKNOWLEDGEMENT

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# *IN VIVO* TRANSFER OF RESISTANCE PLASMIDS IN RAT-, HUMAN- OR PIG-DERIVED INTESTINAL FLORA USING A RAT MODEL

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SUMMARY

*Germ-free rats associated with either rat- (RIF), human- (HIF) or pig (PIF)-derived Enterobacteriaceae-free intestinal flora were used for in vivo experiments to detect transfer of antibiotic resistance.*

*Transfer of resistance was observed most frequently from the porcine donor strain to acceptor strain Escherichia coli K12, showing the highest number of transconjugants in the faeces of HIF-rats. The rats associated with the human donor strain and E. coli K12 as acceptor showed transconjugants less frequently. Only the HIF-rats yielded transconjugants on each sampling day and none at all could be isolated from the PIF-rats. Almost no transconjugants were found in the faeces of rats associated with the pig donor strain and a wild human E. coli strain as acceptor.*

*Factors such as the nature of the donor and recipient strains as well as the origin of the intestinal flora seemed to have an influence on plasmid transfer. Transferability was highest in the HIF-rats and could be increased by administration of lincomycin.*

*This study showed that in vivo transfer of resistance plasmids is possible in rats associated with intestinal floras of different origins. The human intestinal flora seemed to permit better transfer of resistance than that derived from the pig or the rat.*

## INTRODUCTION

Transfer of resistance has been described among species of *Enterobacteriaceae* between animals and even between animals and humans (14,18,20). Contamination with resistant bacteria is not considered to be the major cause of spread of antibiotic resistance between populations, but the carry over of resistance factors (plasmids) has been blamed (12).

As the normal intestinal flora represents a large reservoir of resistance plasmids (11) healthy animals might be expected to constitute a reservoir of resistance genes. Microorganisms might survive in the gut flora and transfer their plasmids to other non-pathogenic or pathogenic intestinal inhabitants or even transfer their resistance to the bacterial flora of other individuals (1). Several studies showed that intensively reared animals such as pigs and chickens have a high incidence of resistant faecal *E. coli* (10,18,21). As infection risks increase when large numbers of animals are concentrated, the quantity of antibiotics used therapeutically and for prevention of bacterial infection is large. Therefore, the faecal *E. coli* pig flora constitutes a possible reservoir of resistance plasmids which might be transferred to humans. This may be a greater public health threat than carriage of pathogenic *Enterobacteriaceae* by animals or the sporadic occurrence of antibiotic residues in meat.

As observed by Corpet (2), *in vitro* transfer results cannot be simply extrapolated to the *in vivo* situation. Therefore, next to *in vitro* conjugation studies, *in vivo* studies are necessary in order to investigate the possibility and extent of plasmid transfer in the presence of the intestinal flora. Germ-free (GF) animals do not harbour a microbial flora and can be colonized with intestinal flora from other species (3,13,19). By keeping these animals in isolators, contamination with environmental bacterial strains or resistance plasmids is avoided and transfer experiments can be executed.

In the present study, GF rats were used for *in vivo* investigation of antibiotic resistance transfer. The aim of this study was to investigate whether *in vivo* transfer of antibiotic resistance to *E. coli* K12 was influenced by the origin of the intestinal flora used. A porcine- and a human-derived donor *E. coli* strain harbouring resistance plasmids, both showing good *in vitro* conjugation activity with *E. coli* K12 (16), were administered to GF rats associated with different *Enterobacteriaceae*-free intestinal floras (human, pig or rat). In addition to these experiments, transfer from the porcine donor strain to a wild human recipient was also examined.

**Animals**

Eighteen male germ-free (GF) rats (WU/Cpb/Bo) of 250-350 g were divided at random into nine groups of two rats and used for three experiments. Three isolators, each housing two rats in a macrolon cage, were used each experiment. The animals were kept and handled in isolators, before and during the experiment, under conditions as required for GF animals (8). The autoclaved drinking water was acidified with HCl to a pH $<3$ except when *E. coli* or antibiotics were added.

**Intestinal floras**

Standard human (5) and standard rat (6) intestinal flora were used. As pig flora free of *Enterobacteriaceae* was not available this was produced following the recommendations of Koopman *et al.* (9). In short: faeces collected from healthy pigs were diluted 1:10 and plated on to anaerobic agar plates (FAA-agar$^R$: fastidious anaerobic agar, LabM no 90, Zoetermeer, The Netherlands) containing 5% sheep blood, haemin (5 mg/l) and menadione (10 mg/l). Colistin (16 mg/l) was added to the plates to inhibit growth of *Enterobacteriaceae*. After incubation for five days at 37°C in an anaerobic glove box in an atmosphere consisting of 80% $N_2$, 10% $CO_2$ and 10% $H_2$, the growth of each plate was harvested, resuspended in approximately 1 ml diluent and used to seed a new agar plate without colistin. In order to check for residual *Enterobacteriaceae* 0.1 ml was spread over a Levine-agar plate (Oxoid CM69). After five days of incubation the *Enterobacteriaceae*-free growth on each plate was harvested, resuspended in 1 ml 10% skim milk (Oxoid L31) and frozen at -70°C until use. All manipulations were performed inside the anaerobic glovebox.

**Bacterial strains**

Two donor and two recipient strains were tested (Table 1). Experiments I and III were performed with *E. coli* donor strain P86. This isolate from a healthy pig was resistant to amoxycillin, chloramphenicol, oxytetracycline, streptomycin, sulphamethoxazole and trimethoprim. *E. coli* donor strain H1803, isolated from a healthy person and showing resistance to amoxycillin, oxytetracycline, streptomycin and sulphamethoxazole, was used in experiment II. Two nalidixic acid resistant (nal$^R$) *E. coli* strains were used as recipients: *E. coli* K12 was used in experiments I and II and *E. coli* H2361, a wild strain isolated from a healthy person, in experiment III. All strains used, except *E. coli* K12, were isolated during another study (17).

**Procedure**

Rats were first colonized with the nalidixic acid resistant recipient *E. coli* (K12 or H2361) by adding 2 ml of an overnight culture (approximately $9x^{10}$log CFU) to 250 ml of non-acidified drinking water which was provided as the only source of water for the rats during 24 hrs. Three days later faeces, were collected and examined for the presence of the recipient strain. On day four the intestinal floras were given by gastric gavage (1 ml). Each group of two rats was housed in a separate isolator. The rats housed in one isolator were given the pig intestinal flora (PIF), two other rats the rat intestinal flora (RIF) and the other two rats received the human intestinal flora (HIF). On day eight faeces were collected for inventarisation of the intestinal flora, including susceptibility testing of the $nal^R$ recipient *E. coli*. Sampling was followed by administration of the donor strain (approximately $9x^{10}$log CFU) in 250 ml of non-acidified drinking water on day 14. Further on, faeces were collected and checked for the presence of transconjugants on days 21, 28 and 32. After faeces collection on day 32 lincomycin (100 mg/l) was added to the drinking water for six days. On day 38 faeces were again collected and lincomycin administration stopped. Finally, faeces were collected on day 47. Faecal samples were collected directly from the anus of each individual rat.

**Bacteriological analysis of the faecal samples**

Nine ml of diluent was added to each gram of faeces in an anaerobic glovebox within 30 minutes of collection of the faeces. Each homogenate was serially diluted tenfold ($10^{-1}$-$10^{8}$) and the (an)aerobic flora calculated. For enumeration of the anaerobic flora the dilutions were inoculated on anaerobic agar plates with and without antibiotics (ciprofloxacin 0.5 mg/l and trimethoprim 5 mg/l) and incubated at 37°C for five days in an anaerobic glovebox in an atmosphere containing 10% $H_2$, 10% $CO_2$ and 80% $N_2$. The bacterial counts on the antibiotic-containing plates were compared with the corresponding plates without antibiotics, in order to check for the growth of facultative anaerobic bacteria.

The total aerobic flora was enumerated on 5% sheep blood agar (Oxoid CM 55). The number of CFU of the donor strain was determined on Iso-sensitest$^R$ agar plates (Oxoid CM471) containing a selective antibiotic. The agents tested and the concentrations used were as follows: amoxycillin (32 mg/l), chloramphenicol (32 mg/l), oxytetracycline (32 mg/l), streptomycin (20 mg/l), sulphamethoxazole (256 mg/l) and trimethoprim (4 mg/l).

The recipient and transconjugant strains were selected on plates containing nalidixic acid (32 mg/l) either alone or in combination with one of previously mentioned antibiotics. To prevent overgrowth by Gram-positive bacteria vancomycin (7.5 mg/l) was added to all

*Table 1: Mean counts (from two rats in the same isolator) of donor and recipient Escherichia coli strains in the three experiments after five faeces collections on days 21, 28, 32, 38 and 47 (in $^{10}$log CFU/g faeces).*

| | | DONOR mean SD | RECIPIENT mean SD |
|---|---|---|---|
| **Experiment I** | | P86 | K12 |
| | PIF | 8.6 ± 0.3 | 7.0 ± 0.3 |
| | RIF | 8.2 ± 0.3 | 6.8 ± 0.2 |
| | HIF | 8.6 ± 0.5 | 5.8 ± 0.4 |
| **Experiment II** | | H1803 | K12 |
| | PIF | 8.5 ± 0.3 | 5.2 ± 0.5 |
| | RIF | 8.8 ± 0.5 | 4.2 ± 0.4 |
| | HIF | 9.0 ± 0.2 | 5.3 ± 0.5 |
| **Experiment III** | | P86 | H2361 |
| | PIF | 7.9 ± 1.1 | 7.3 ± 0.5 |
| | RIF | 8.1 ± 0.6 | 7.9 ± 0.4 |
| | HIF | 7.6 ± 0.7 | 7.7 ± 0.6 |

*SD= standard deviation, PIF/RIF/HIF= pig/rat/human intestinal flora associated rats. P86= wild porcine donor strain, H1803= wild human donor strain, K12= E. coli K12 recipient strain, H2361= wild human recipient strain.*

agar plates. The plates were incubated for 24-48 hrs at 37°C in air. In addition, each of the $10^{-1}$ diluted faecal samples (1 ml) was cultured overnight under antibiotic pressure (256 mg/l sulphamethoxazole and 32 mg/l nalidixic acid) in 5 ml BHI (brain heart infusion broth), and when no transconjugants were found the next day these overnight cultures were inoculated on to antibiotic-containing plates for transconjugant detection. If transconjugants were isolated from these agar plates, this is represented as + in Tables 2 and 3. *E. coli* identification and susceptibility testing was performed as previously described (15). The identification of the first 50 *E. coli* isolates was confirmed with Api-20E biochemical system (BioMerieux, Den Bosch, The Netherlands). The transfer frequency was calculated as the ratio of the number of transconjugants to the number of recipients. The number of transconjugants per rat were counted as the mean number of transconjugants present on all selective plates tested (Figure 1). Aerobic and anaerobic counts of the faecal flora of SPF (specified pathogen free) WU-rats were determined as controls.

**Plasmid isolation**

Plasmid DNA was extracted by a modification of the method described by Kado & Liu (7). The molecular weights of the plasmids were determined by agarose gel electrophoresis using 0.7% agarose gel and running at 75V for four hours. Ethidiumbromide was used to visualize DNA with ultraviolet light after the run was completed. A *Salmonella typhimurium* strain (kindly provided by Dr. N. van Leeuwen, RIVM) containing five plasmids (91, 39, 7.6, 5.8 and 4.4 Kb) was used as reference.

## RESULTS

*In vitro*

The mean frequency of transfer between porcine donor strain P86 and recipient *E. coli* K12 *in vitro* was $2.3 \times 10^{-4}$. A lower frequency of transfer was observed with recipient H2361 ($5.5 \times 10^{-6}$). The human donor strain H1803 showed a frequency of transfer to *E. coli* K12 similar to that of P86 ($2.3 \times 10^{-4}$) except for oxytetracycline for which no transfer at all was found.

*In vivo*

All bacterial counts are given in $^{10}$log CFU/g faeces. The minimum transconjugant detection level was 2.0 $^{10}$log CFU/g faeces. The total aerobic counts of the two control rats were 7.7 and 7.8 and the total anaerobic counts 9.0 and 9.1.

In GF rats colonized only with a recipient *E. coli* strain the number of *E. coli* per gram faeces was constant ($8.9 \pm 0.2$) independent of the recipient used. After establishment of the intestinal floras the numbers of *E. coli* K12 per gram faeces decreased to approximately $6.8 \pm 1.0$, whereas for the wild-recipient strain H2361 no such decline could be observed ($8.6 \pm 0.5$) either in PIF-, RIF- or HIF-rats. All rats showed rather constant total anaerobic ($9.4 \pm 0.6$) and aerobic counts ($8.5 \pm 0.7$). Both donor strains showed good colonization in the different intestinal floras (Table 1). As the rats kept in the same isolator showed minimal variation in CFU/g faeces of donor and recipient strains, the means of two rats per isolator are presented in Table 1.

After establishment of the donor strains, but before lincomycin administration, the number of anaerobes and aerobes remained at the same level as before in all rats. A slight increase (approximately 1 log) in total aerobic counts was observed in all experiments after lincomycin administration. In contrast, the total anaerobic counts decreased after

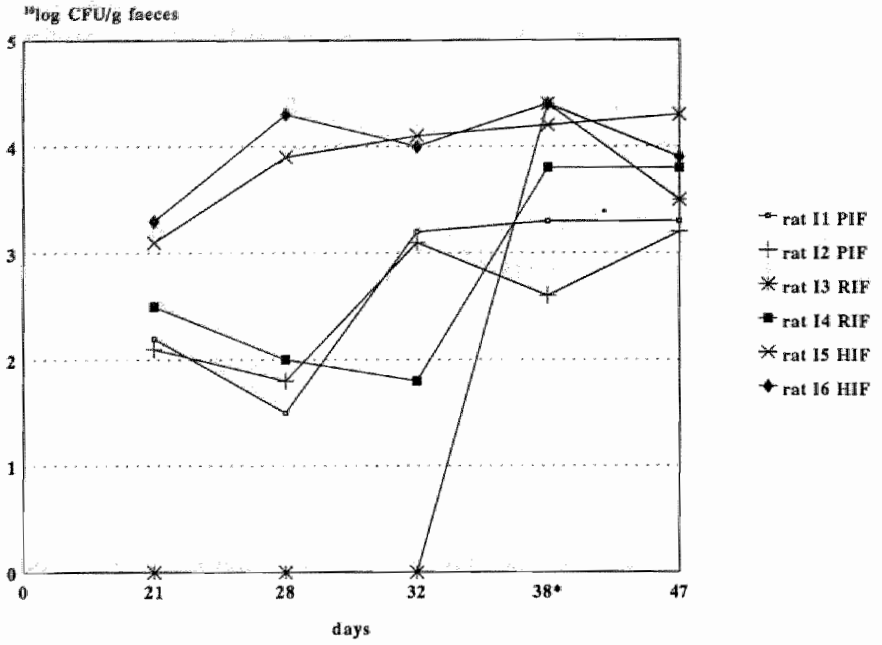


*Figure 1: Mean number of transconjugants isolated from GF rats associated with pig-, rat- or human-derived Enterobacteriaceae-free intestinal floras (experiment I). PIF/RIF/HIF-rats= pig/rat/human intestinal flora associated rats, *= lincomycin administered.*

lincomycin administration (approximately 4 logs), but returned to normal levels one week after the administration of this drug was stopped. The numbers of donor strains were similar for each flora in each experiment, except that a possible influence of lincomycin on the donor counts could be observed in the PIF-rats (Table 1) in experiment III. The number of recipients differed per experiment. Before lincomycin administration, recipient *E. coli* K12 was present in lower numbers than recipient H2361 (experiment III). The lowest number of CFU/g faeces was found in experiment II for recipient *E. coli* K12, especially in the RIF-rats.

On days 21, 28 and 32 (thus before lincomycin administration), the number of recipient *E. coli* K12 strains in experiment I ranged from 6.9 to 7.0 for the PIF-rats and from 6.6 to 7.0 for the RIF-rats, but decreased approximately one log for the HIF-rats (5.9-6.2). The numbers of recipient *E. coli* K12 strains in experiment II before lincomycin administration ranged between 4.6 and 5.4 in the PIF-rats, 4.1 and 4.5 in the RIF-rats and between 5.1 and 5.8 in the HIF-rats. In experiment III the numbers of recipients per intestinal flora were of the same order as the numbers of donor strain P86 (Table 1). Lincomycin administration seemed to be of no influence on the numbers of recipient strains.

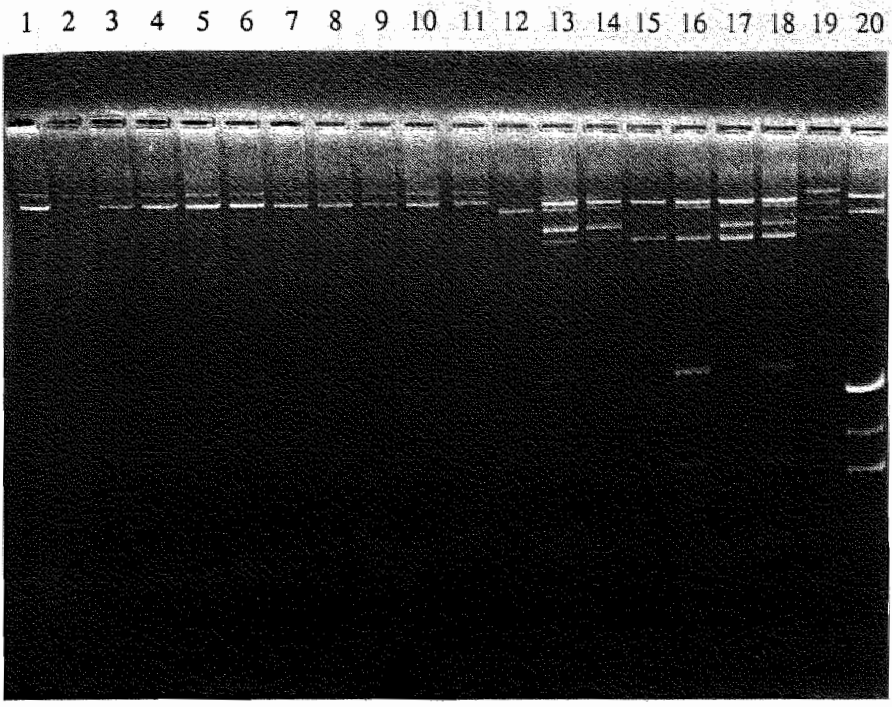


*Figure 2: Agarose gel electrophoresis of plasmid DNA extracted from porcine and human E. coli donor strains (D) and some transconjugants (T) . Lane 1-8 T of experiment I, resistance pattern amoxycillin-chloramphenicol-oxytetracycline-streptomycin-sulphamethoxazole-trimethoprim (AmxCOtSSmxTmp); lane 9-10 T of experiment III (AmxCOtSSmxTmp); lane 11 D P86 (AmxCOtSSmxTmp); lane 12 T of experiment II (SSmx); lane 13-14 T of experiment II (AmxOtSSmx); lane 15-18 T of experiment II (AmxSSmx); lane 19 D H1803 (AmxOtSSmx). In lane 20 the reference strain Salmonella typhimurium with size standards in descending order 91, 39, 7.6, 5.8 and 4.4 Kb is extracted.* ***Footnote:*** *unfortunately the second plasmid of the transconjugant in lane 2 is not visible on this figure, however it was slightly visible on the gel.*

## Transconjugants

*Experiment I:* donor *E. coli* P86, recipient *E. coli* K12.

On day 21, transconjugants could be observed for all rats, except for rat I3 (Figure 1). Both HIF-rats showed the highest number of transconjugants and these could be selected on each selective plate. Remarkably, before lincomycin administration, one RIF-rat (I4) showed transconjugants at each sampling whereas the other one (I3) showed none at all. After lincomycin administration, transconjugants were observed in all rats, showing the highest rates for the HIF-rats and the lowest rates for the RIF-rats. All transconjugants

*Table 2: Number of transconjugants (in $^{10}$log CFU/g faeces) isolated from* ***experiment II****: donor E. coli H1803, recipient E. coli K12.*

| day | 21 | | 28 | | 32 | | 38* | | 47 | |
|---|---|---|---|---|---|---|---|---|---|---|
| **PIF** rat nr. | II1 | II2 | II1 | II2 | II1 | II2 | II1 | II2 | II1 | II2 |
| Amx | - | - | - | - | - | - | - | - | - | - |
| Ot | - | - | - | - | - | - | - | - | - | - |
| S | - | - | - | - | - | - | - | - | - | - |
| Smx | - | - | - | - | - | - | - | - | - | - |
| **RIF** rat nr. | II3 | II4 | II3 | II4 | II3 | II4 | II3 | II4 | II3 | II4 |
| Amx | - | 2.0 | - | + | - | - | - | + | - | - |
| Ot | - | + | - | + | - | - | - | + | - | - |
| S | - | - | - | - | - | - | - | + | - | - |
| Smx | - | + | - | + | - | + | - | + | - | + |
| **HIF** rat nr. | II5 | II6 | II5 | II6 | II5 | II6 | II5 | II6 | II5 | II6 |
| Amx | 3.9 | 3.8 | 3.1 | 3.0 | 3.1 | 2.9 | 3.8 | 3.5 | 4.0 | 2.5 |
| Ot | + | - | + | + | + | + | + | + | + | - |
| S | 3.0 | 3.0 | 2.9 | + | 2.3 | 2.3 | 3.9 | 2.5 | 3.7 | 2.3 |
| Smx | 3.6 | 3.5 | 3.5 | 3.2 | 3.0 | 2.5 | 3.6 | 3.2 | 4.1 | 3.0 |

*day= sampling day; *= lincomycin administrated; PIF/RIF/HIF-rats= pig/rat/human intestinal flora associated rats; rat nr.= rat number. Amx= amoxycillin, Ot= oxytetracycline, S= streptomycin, Smx= sulphamethoxazole. -= absence of transconjugants after overnight selection under antibiotic pressure, += presence of transconjugants after overnight selection under antibiotic pressure, but not after overnight incubation without antibiotic selective pressure. Detection limit transconjugants: 2.0 $^{10}$log CFU/g faeces.*

showed the same resistance pattern as the donor strain. The two large plasmids observed in the donor strain were present in all transconjugants (Figure 2). A third small plasmid was only present in some transconjugants.

In *experiment II*, using donor *E. coli* H1803, recipient *E. coli* K12 transconjugants were observed in all HIF-rats (Table 2). In contrast, only one of the two RIF-rats showed minimal transfer of resistance and no transfer at all was found in the PIF-rats. Almost all transconjugants showed resistance to amoxycillin-oxytetracycline-streptomycin-sulphamethoxazole and amoxycillin-streptomycin-sulphamethoxazole, some strains were resistant to streptomycin-sulphamethoxazole. The plasmid profile of the transconjugants resistant to amoxycillin-oxytetracycline-streptomycin-sulphamethoxazole was the same as for the donor strain. Strains with amoxycillin-streptomycin-sulphamethoxazole or streptomycin-sulphamethoxazole showed only part of the donor plasmid profile (Figure 2).

*Table 3: Number of transconjugants (in $^{10}$log CFU/g faeces) isolated from experiment III: donor E. coli P86, recipient E. coli HR2361.*

| day | | 21 | | 28 | | 32 | | 38* | | 47 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| PIF rat nr. | | III1 | III2 | III1 | III2 | III1 | III2 | III1 | III2 | III1 | III2 |
| | Amx | + | + | + | - | - | - | - | - | - | - |
| | C | + | + | 4.1 | + | - | - | - | - | - | - |
| | Ot | - | + | - | - | - | - | - | - | - | - |
| | S | - | - | - | - | - | - | - | - | - | - |
| | Smx | - | + | + | - | - | - | - | - | - | - |
| | Tmp | + | + | - | - | - | - | - | - | - | - |
| RIF rat nr. | | III3 | III4 | III3 | III4 | III3 | III4 | III3 | III4 | III3 | III4 |
| | Amx | - | - | - | - | - | - | - | + | - | + |
| | C | - | - | - | - | - | - | - | + | - | + |
| | Ot | - | - | - | - | - | + | - | + | - | + |
| | S | - | - | - | - | - | + | - | + | - | + |
| | Smx | - | - | - | - | - | + | - | + | - | + |
| | Tmp | - | - | - | - | - | + | - | + | - | + |
| HIF rat nr. | | III5 | III6 | III5 | III6 | III5 | III6 | III5 | III6 | III5 | III6 |
| | Amx | - | - | - | - | - | - | - | - | - | - |
| | C | - | - | - | - | - | - | - | - | - | - |
| | Ot | - | - | - | - | - | - | - | - | - | - |
| | S | - | - | - | - | - | - | - | - | - | - |
| | Smx | - | - | - | + | - | - | - | - | - | - |
| | Tmp | - | - | - | - | - | - | - | - | - | - |

*C= chloramphenicol, Tmp= trimethoprim; see also legend Table 2.*

In *Experiment III*, using donor *E. coli* P86 and recipient *E. coli* R2361, almost no transfer of resistance was observed (Table 3). Only once were PIF-rat transconjugants observed in a sample without overnight incubation under antibiotic pressure. After overnight selection under antibiotic pressure, transconjugants were isolated most frequently from PIF-rats, but only once from a HIF-rat. No differences were observed before and after lincomycin administration. Also in this experiment only one RIF-rat showed transfer of resistance. All transconjugants showed the same resistance pattern as the donor strain. The two large plasmids observed in the donor strain were present in all transconjugants (Figure 2). A third small plasmid was only present in some transconju-

gants.

## DISCUSSION

In the present study *in vivo* plasmid transfer from wild porcine and human *E. coli* strains to *E. coli* K12 was observed. Moreover the data strongly suggest that *in vivo* transfer of antibiotic resistance is influenced by the donor and recipient used as well as the colonization resistance (22) of the intestinal flora. The time-schedule chosen, i.e. day of administration of the intestinal floras and donor strains and of collecting faecal samples, was based on data obtained in a pilot study which showed clearly that after these time intervals the bacterial counts of recipient, anaerobic and aerobic bacteria remained constant.

After associating the rats with an intestinal flora the concentration of recipient *E. coli* K12 decreased in the faeces, which might be due to factors such as competition for substrates and for adhesion-sites in the intestine or the production of growth inhibitors (4). However competition or inhibition seemed to be of minor influence for both wild donor strains.

Although rats associated with a homologous rat flora (RIF) were expected to have an optimal colonization resistance, both wild porcine and human donor strains (P86, H1803) could colonize these floras in high numbers in all three experiments. In contrast, however, the transfer activity in these rats (RIF) was less than in the HIF-rats (exp. I and II).

One might expect that rats housed in the same cage would become colonized with the same flora. However, one RIF-rat in experiment I showed no transfer before lincomycin administration although the other one did (Figure 1). The intestinal flora of this rat seemed to prevent transfer of resistance and colonization by transferred strains. Indeed, after disturbing this flora with lincomycin the transfer rate was comparable to that of the human flora. No obvious increase in CFU of transconjugants/g faeces was observed in the HIF-rats of experiment I after lincomycin administration. This finding might be explained by the already high number of transconjugants in relation to the number of recipient *E. coli* K12.

The observations in the present study strongly suggest that the intestinal flora influences the frequency of transfer. The rat intestinal flora inhibited transfer more efficiently than the human intestinal flora. This is probably due to a better colonization resistance provided by a species-specific flora.

The differences in transferability observed in experiments I and II might be due to the use of different donor strains. Although with both donor strains *in vitro* transfer was observed

as well as *in vivo* colonization of the rats in high numbers, differences in transfer activity were recorded. A higher frequency of transfer was observed for the porcine donor strain P86 compared to the human strain H1803. Thus P86 seemed to be a better donor of plasmids than the human strain H1803. A second difference was the lower numbers of recipient strain *E. coli* K12 that were able to colonize the rats in experiment II. In addition, resistance transfer in both experiments was most frequently observed in the presence of the human flora, thus suggesting that this flora can pick up pig-derived resistance plasmids. Comparing experiments I and III (the same donor strain), clear differences in transferability were observed which might be related to the use of different recipients.

As expected, lincomycin administration caused a reduction in the growth of anaerobic bacteria, however no increase in the numbers of donor or recipient *E. coli* strains was observed. After lincomycin administration, no transconjugants were observed for the PIF- rats in experiments II and III in neither of which could transconjugants be isolated on day 32. No influence on the frequency of transfer in the RIF rats in experiments II and III was observed after lincomycin administration and lincomycin seemed only to be of minor influence in these experiments. Into account the data obtained, one has to realize that no detection of transconjugants in the faeces does not prove absence of plasmid transfer and it is possible that the numbers of transconjugants present were below the detection level.

In conclusion, *in vivo* transfer of antibiotic resistance is possible using GF rats associated with different sorts of intestinal flora. In general, the highest frequency of transfer was observed when the rats were colonized with human flora. This might be due to the fact that human flora permits better transfer of resistance. However, more and extended experiments are necessary.

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# SURVEILLANCE OF ANTIBIOTIC RESISTANCE AMONG PIGS USING FAECAL FLOOR DROPPINGS FROM TRUCKS TRANSPORTING FATTENING PIGS

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SUMMARY

*Faecal samples from farm pigs (n=291) and faecal floor droppings from trucks, transporting fattening pigs to the slaughterhouse in the same region (n=292), were analyzed for prevalence and degree of antibiotic resistance in Escherichia coli. In addition, the antibiotic susceptibility of E. coli isolated from both groups was determined.*

*The aim of this study was to examine whether, instead of the cumbersome and time consuming collection of individual rectal faeces samples from pigs, floor droppings collected from trucks transporting fattening pigs could be used for resistance surveillance. Both the prevalence and high degree of resistance observed were of the same size of order. This was also observed for susceptibility testing and the $MIC_{50/90}$ distribution. Most frequently isolated resistance patterns for both groups were oxytetracycline-streptomycin and oxytetracycline-streptomycin-sulphamethoxazole .*

*From this study it appears that faecal floor droppings collected from trucks transporting fattening pigs are useful for antibiotic surveillance and therefore can be used for detection of changes in antibiotic resistance in the faecal flora of a pig population. Consequently, continuous surveillance of resistance seems feasible.*

## INTRODUCTION

There is a growing concern about the increase of antibiotic resistance in pathogenic bacteria mainly as a consequence of extensive use of antibiotics both in human and veterinary medicine (8,13,19). As resistance develops in rough relation to antibiotic use the best way to minimise emergence of resistance, without hampering optimal care, is to minimise the use of antibiotics by using them only if they are really indicated. This can be reached by developing an antibiotic policy and an antibiotic formulary aimed to improve rational treatment of bacterial infections and minimizing emergence and spread of resistant bacteria (2,14). Different formularies can be written for different populations e.g. for medical antibiotic use by patients and for veterinary use by different groups of animals in a certain province or country. Implementation of an antibiotic policy should be followed by monitoring the use of antibiotics and by surveillance of antibiotic resistance in the target population, not only of isolated pathogenic bacteria but of the non-pathogenic faecal flora as well (2,22).

As intestinal bacteria carrying resistance factors are wide-spread in the environment (16,19,23) the faecal flora in humans and animals is considered to be a good indicator for the regular monitoring of antibiotic resistance in a population (1,11,12,24). However, regular sampling of large numbers of individual pig faecal samples collected at farms is laborious, time-consuming and very expensive. Hence, this method is not suitable for a continuous resistance surveillance.

This study was undertaken to answer the question whether faecal droppings collected from trucks are indicative for the faecal bacterial resistance in pigs living in the same region at different farms. If in faecal samples collected from the floor of trucks transporting fattening pigs the antibiotic resistance of *Escherichia coli* is of the same size of order as in the regional pig population this material could be used for resistance surveillance on a regular or even continuous base. The prevalence and high degree of resistance of the isolates of both groups as well as the susceptibility were determined and compared. As the prevalence of resistance strongly depends on the method used the high degree of resistance is considered to be a more reliable and relevant parameter for resistance surveillance than the prevalence of resistance (3,4,17). The probability of spread of antibiotic resistant bacteria or resistance genes to susceptible strains is higher in animals of which a majority of the faecal *Enterobacteriaceae* flora is resistant (i.e. high degree of resistance) than in animals with low numbers of antibiotic resistant strains (i.e. low degree of resistance).

**Collection of the faecal samples**

Fresh faecal samples from two different groups of pigs living in the same region were collected. One group, described in a previous study (21), consisted of mixed faecal samples (n=291) from mature gilts and/or heavy porkers collected per rectum at pig farms. Only one sample per farm was analyzed. The second group consisted of floor dropping samples (n=292) from trucks transporting fattening pigs from different farms. Immediately after delivering the pigs at the slaughterhouse two samples were taken from each truck. After collecting, the samples were sent to the bacteriological laboratory where they were diluted ($10^{-1}$) in 0.9% saline, containing 20% (v/v) glycerol and stored at -20°C until examined.

**Bacteriological analysis of the faecal samples**

To compare the results of both methods of faeces collection the prevalence and degree of resistance as well as the susceptibility and $MIC_{50/90}$ were determined. However, if truck sampling is an useful alternative for sampling individual pigs the data, especially the high degree of resistance, obtained with both methods must be of the same size of order.

The methods used to determine the prevalence and degree of antibiotic resistance and the antibiotic susceptibility were as described previously (20). The breakpoint concentrations used for determining antibiotic susceptibility are shown in Table 2.

## RESULTS

The data observed for the farm pigs have been presented in a previous study (21).

Sixty-nine and 93% of the colonies tested isolated from farm pig and floor dropping samples, respectively, with the morphology typical for *E. coli* on Levine-agar were identified as *E. coli*. The other colonies tested were also *Enterobacteriaceae*: *Klebsiella* spp., *Citrobacter* spp., *Enterobacter* spp. and *Proteus* spp. All faecal farm pig samples showed growth on the agar plates without antibiotics, whereas 6 of the 292 floor droppings failed to grow. *E. coli* could not be isolated from four farm pig samples and 11 floor droppings because of overgrowth by *Bacillus* spp. In addition, from two farm pig samples and 27 floor droppings, respectively, the colonies with the morphology of *E. coli* isolated were indole negative and excluded for susceptibility testing. Finally 285 and 248 *E. coli* strains isolated from faecal samples collected at farms and from trucks, respective-

ly, were tested for susceptibility.

*Prevalence of antibiotic resistance*

The prevalence of resistance to neomycin, amoxycillin, trimethoprim, oxytetracycline and sulphamethoxazole ranged between 92% and 100% for the farm pigs and between 73% and 89% for the floor droppings (Table 1). The prevalence of resistance to the other antimicrobial agents tested was distinctly lower for both groups.

*Degree of antibiotic resistance*

For both groups of faecal samples examined a high degree of resistance was observed for the antimicrobial agents commonly used in veterinary medicine i.e. amoxycillin, neomycin, oxytetracycline, sulphamethoxazole and trimethoprim (Table 1). The highest percentages in farm pigs and floor droppings were found for oxytetracycline and sulphamethoxazole. For the other compounds the percentages were distinctly lower.

For both groups of samples comparable high degree of resistance percentages were observed with exception of the floor droppings, which showed a considerably higher

*Table 1: Prevalence and high degree of antibiotic resistant Escherichia coli in pig faeces collected at pig farms (n=291) and from trucks transporting fattening pigs (n=286).*

| Antibiotic | | Prevalence | | High degree | |
|---|---|---|---|---|---|
| | | Farms | Trucks | Farms | Trucks |
| | mg/l^ | % | % | % | % |
| Amx | 25 | 98 | 84 | 11 | 12 |
| Ap * | 32 | 8 | 1 | 0 | 0 |
| Cip | 4 | 2 | 0 | 0 | 0 |
| Flu | 4 | 2 | 1 | 0 | 0 |
| Ft | 50 | 17 | 4 | 0 | 0 |
| Ne | 8 | 92 | 73 | 2 | 2 |
| Ot | 25 | 100 | 89 | 25 | 45 |
| Smx | 100 | 100 | 89 | 24 | 27 |
| Tmp | 8 | 99 | 77 | 7 | 3 |

*mg/l^: Antibiotic concentrations in the agar plates, *: Apramycin was only tested for the last 144 faecal samples of the farm pigs and for all truck samples. Amx= amoxycillin, Ap= apramycin, Cip= ciprofloxacin, Flu= flumequin, Ne= neomycin, Ft= nitrofurantoin, Ot= oxytetracycline, Smx= sulphamethoxazole, Tmp= trimethoprim.*

*Table 2: Antibiotic resistance of Escherichia coli strains isolated from faecal pig samples collected at farms and from trucks transporting fattening pigs.*

| Antibiotic | | Antibiotic resistant *E. coli* strains | |
|---|---|---|---|
| | mg/l^ | **Farms** (n=285) n (%) | **Trucks** (n=248) n (%) |
| Amx | 16 | 72 (25) | 50 (20) |
| Ap | 16 | 0 (0) | 0 (0) |
| Amc | 16 | 0 (0) | 0 (0) |
| C | 8 | 38 (13) | 40 (16) |
| Ft | 32 | 23 (8) | 10 (4) |
| Gm | 4 | 3 (1) | 4 (2) |
| Na | 8 | 1 (0) | 2 (1) |
| Ne | 16 | 20 (7) | 15 (6) |
| Ot | 16 | 163 (57) | 171 (69) |
| S | 16 | 201 (71) | 208 (84) |
| Smx | 128 | 129 (45) | 118 (48) |
| Tmp | 2 | 46 (16) | 31 (13) |

*mg/l^: breakpoint concentration, according to Klingeren et al. (7) and for apramycin according to Hunter (6). Amc= amoxycillin+clavulanic acid, C= chloramphenicol, Gm= gentamycin, Na= nalidixic acid, S= streptomycin. See also legend Table 1.*

*Table 3: Most frequently isolated patterns of drug resistant Escherichia coli strains isolated from faecal pig samples collected at farms and from trucks transporting fattening pigs.*

| Resistance Pattern | | Percentage of *E. coli* strains **Farms** (n=285) n (%) | **Trucks** (n=248) n (%) |
|---|---|---|---|
| | Ot S | 42 (15) | 53 (21) |
| | Ot S Smx | 27 (10) | 35 (14) |
| | S Smx | 21 (7) | 12 (5) |
| | S | 15 (5) | 29 (12) |
| Amx | Ot S Smx Tmp | 9 (3) | 8 (3) |

*Legend see Table 1*

percentage of oxytetracycline resistance.

*Antibiotic resistance*

Both groups showed the highest resistance percentages for sulphamethoxazole, oxytetracycline and streptomycin ranging between 45%-71% and 48%-84% for farm pigs and floor droppings, respectively (Table 2). Seventy different resistance patterns could be distinguished for the farm pigs and 50 for the floor droppings. Most of these patterns (46 farm pig and 31 floor dropping samples) were observed only once or twice. The three most frequently recorded resistance patterns in both groups were a combination of streptomycin and/or oxytetracycline and/or sulphamethoxazole (Table 3). These patterns did occur more or less with the same frequency in both populations. Only resistance to streptomycin alone seemed to be higher in the floor dropping isolates (12%, versus 5%). The $MIC_{50}$ and $MIC_{90}$, considered to be the best predictive values for clinical efficiency, were also similar for both groups (Table 4).

*Table 4: MIC50 (mg/l) and MIC90 (mg/l) of Escherichia coli strains isolated from faecal pig samples collected at farms and from trucks transporting fattening pigs.*

| | Farms (n=285) | | Trucks (n=248) | |
|---|---|---|---|---|
| Antibiotic | MIC50 | MIC90 | MIC50 | MIC90 |
| Amx | 8 | ≥64 | 4 | ≥64 |
| Ap | 4 | 8 | 8 | 8 |
| Amc | 4 | 16 | 4 | 8 |
| C | 8 | 16 | 8 | 64 |
| Gm | 0.5 | 1 | 1 | 2 |
| Na | 4 | 8 | 4 | 8 |
| Ne | 2 | 4 | ≤1 | 8 |
| Ft | 16 | 32 | 16 | 32 |
| Ot | ≥256 | ≥256 | ≥256 | ≥256 |
| S | 32 | ≥128 | 64 | ≥128 |
| Smx | 64 | ≥1024 | 64 | ≥1024 |
| Tmp | 0.5 | 512 | 0.5 | 512 |

*MIC50= Minimal Inhibitory Concentration (mg/l) at which 50% of the strains are inhibited, MIC90= 90% inhibited. See also legend Tables 1 and 2.*

The present study showed that the prevalence and high degree of resistance to most compounds tested were for both groups of faecal samples of the same size of order. In addition, the antibiotic resistance percentages of the *E. coli* isolated, the $MIC_{50/90}$ distribution as well as the resistance patterns most frequently observed were of the same size of order in both groups. No explanation for the higher percentage of high degree of resistance to oxytetracycline observed in the floor droppings could be found. However, in a previous study about monitoring of antibiotic resistance in fattening pigs on a single farm the high degree of resistance to oxytetracycline varied over time as well (20). Differences between both groups were found in percentage indole negative samples and samples with overgrowth by *Bacillus* spp. The floors of the trucks were probably contaminated with a high diversity of soil bacteria, resulting in more difficulties with bacterial overgrowth and indole negative strains during processing these samples.

Continuous surveillance of antibiotic resistance of pathogenic and non-pathogenic micro-organisms is an essential part of an optimal antibiotic policy. The faecal flora is considered to be an important reservoir of antibiotic resistant genes and thus useful for antibiotic resistance monitoring of the healthy animal or human population (12,15,24,25). Therefore, an easy method to obtain indicative faecal samples at regular intervals is required. Smith (24) investigated individual faecal samples of pigs at markets, during 1956 and 1970-1979, to reflect the occurrence of antibiotic resistance in the pig population. In The Netherlands trading of pigs at markets is strongly discouraged, because of spread of infectious diseases prevention. In this study floor droppings from trucks transporting pigs for slaughter were collected as an alternative for individual pig faecal samples. The data of the present study strongly suggest that the alternative for antibiotic resistance surveillance investigated in this study is useful and relatively easy to perform. Therefore, this method could make more regular or even continuous resistance surveillance in pigs feasible. We are aware of the fact that this method does not take into account differences in factors such as pig age, husbandry methods, particular health problems, nutrition, preventive and therapeutic antibiotic use (5,9,10) which are present at different farms. However, these differences are for surveillance of antibiotic resistance in a certain region or country less important. The data obtained are essential for resistance epidemiology, necessary for an antibiotic policy, that aims to minimise the emergence and spread of antibiotic resistance.

To prevent problems caused by contamination with soil bacteria, it might be advisable to collect faecal samples at the evisceration-line in the slaughterhouse instead of floor

droppings from the trucks.

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GENERAL DISCUSSION

GENERAL CONCLUSIONS AND RECOMMENDATIONS

# GENERAL DISCUSSION

This thesis describes the prevalence and degree of antibiotic resistance in the faecal *E. coli* population of three groups of pigs and human volunteers living in the same area. Additionally, one *E. coli* isolate was randomly selected from each faecal pig and pig farmer sample and the susceptibility for several antibiotics was determined as minimum inhibitory concentrations. The three groups of human participants i.e. pig farmers, abattoir workers and (sub)urban residents, differed in their intensity and frequency of contact with pigs or pig products. Special attention was paid to recent usage of antibiotics by the participants themselves and other persons belonging to the same household, or for treatment of pigs at the different farms. In addition to the study of these phenotypic resistance properties, a selected group of pairs of *E. coli* strains, isolated from pig farmers and their pigs, were compared for genotypic resistance markers: transferability of antibiotic resistance *in vitro* and *in vivo* and characterization of the plasmid patterns of donor and transconjugants using DNA-fingerprinting.

In this chapter, first the results of the pig samples are compared followed by the results of the human samples and a possible relation between the presence of antibiotic resistance in both groups is discussed. Next, the importance of surveillance of antibiotic resistance in pigs is quoted and, finally, general conclusions and recommendations are made.

The faecal flora is an important reservoir of antibiotic resistant *E. coli* in a population, which might transfer resistance plasmids to pathogenic bacteria in that population. Therefore, a high prevalence of antibiotic resistance in the intestinal flora is considered to constitute a public health risk. If the human intestinal tract can be colonized by resistant bacteria or if bacteria from pigs can transfer their resistance genes to the human intestinal flora, measures to prevent this are indicated, including lowering the resistance in the intestinal flora of pigs and/or hygienic measures preventing contact of humans with pig bacteria. However, referring to our results the effect of these measures could be questioned.

## Antibiotic resistance in pigs

Faecal samples of three groups of pigs were analyzed. First, during a period of about one year at one fattening farm, faecal samples were collected at regular intervals from several pigs in the same and in different compartments in order to study the reproducibility of

sampling and the variation in resistance, and to monitor the percentage and degree of resistance over time. The second group studied were faecal samples of pigs from the pig farmers participating in this study and the third group of faecal samples consisted of floor droppings collected from trucks that had transported fattening pigs from farms in the study area to the slaughterhouse.

As shown in chapter II, only minimal variation in prevalence of resistance in the faeces of pigs from the same and from different compartments at the same farm was found. The observed prevalence of resistance did not vary much with time, suggesting that antibiotic resistance in a pig population is relatively constant. Therefore, the mean prevalence of resistance percentage was calculated for all pigs investigated during the 11 month period (Table 1).

In all three pig groups investigated in this study the faecal samples showed the same order of magnitude for the prevalence of resistance, except for neomycin (Table 1). A certain variation in the prevalence of neomycin resistance had also been observed in the faecal samples of groups of pigs from the same fattening farm (chapter II). No explanation could be found for this relatively high variation in resistance to neomycin observed in these

*Table 1: Comparison of the prevalence (%) and high degree (%) of resistant Escherichia coli isolated from different groups of pigs described in present thesis.*

| | **Prevalence of resistance (%)** | | | **High degree of resistance (%)** | | |
|---|---|---|---|---|---|---|
| chapter | II | IV | VII | II | IV | VII |
| n= | 407 | 291 | 286 | 407 | 291 | 286 |
| | X (SD) | | | X (SD) | | |
| Amx | 81 (17) | 98 | 84 | 10 (12) | 11 | 12 |
| Ap | 1 (2) | 8 | 1 | 0 (0) | 0 | 0 |
| Cip | 0 (0) | 2 | 0 | 0 (0) | 0 | 0 |
| Flu | 0.3 (1) | 2 | 1 | 0 (0) | 0 | 0 |
| Ft | 8 (13) | 17 | 4 | 0 (0) | 0 | 0 |
| Ne | 51 (25) | 92 | 73 | 1 (4) | 2 | 2 |
| Ot | 94 (10) | 100 | 89 | 32 (20) | 25 | 45 |
| Smx | 94 (11) | 100 | 89 | 17 (13) | 24 | 27 |
| Tmp | 88 (18) | 99 | 77 | 3 (4) | 7 | 3 |

*Chapter II= different groups of pigs living at a single farm, chapter IV= pigs living at different farms, chapter VII= floor droppings collected from trucks transporting fattening pigs. Amx= amoxycillin, Ap= apramycin, Cip= ciprofloxacin, Flu= flumequin, Ft= nitrofurantoin, Ne= neomycin, Ot= oxytetracycline, Smx= sulphamethoxazole, Tmp= trimethoprim, X= mean of the samples of pigs described in chapter II, SD= standard deviation, n= number of samples tested.*

studies. The use of neomycin by the pig breeders might be a possible explanation, but no information about this use could be obtained.

A high degree of resistance was observed for antibiotics commonly used for pig treatment in veterinary medicine: amoxycillin, oxytetracycline, sulphamethoxazole, trimethoprim and neomycin. A variation in high degree of resistance for oxytetracycline could be observed.

The resistance percentages observed in the three pig groups were of the same order of magnitude (Table 2). Also, the most frequently present resistance patterns, i.e. a combined resistance against oxytetracycline and/or sulphamethoxazole and/or streptomycin, were observed in all three groups of pigs (Table 3). The high resistance percentages to oxytetracycline and sulphamethoxazole found might be explained by the fact that these compounds are the most commonly used antibiotics for mass-medication of pigs in The Netherlands (2). Streptomycin, however, is currently only used in pigs for individual and parenteral therapy in combination with penicillin but, despite this, high resistance percentages were observed for this agent. As genes encoding resistance to streptomycin are in most cases located on plasmids and frequently in combination with other genes conferring

*Table 2: Resistance percentages (%) observed for pigs in present thesis and by other investigators.*

| | **Present thesis** | | | | **Other studies** | | | |
|---|---|---|---|---|---|---|---|---|
| chapter | II | IV | VII | ref | 1 | 3 | 7 | 14 |
| n= | 387 | 285 | 248 | n= | 53 | 5 | 359 | 796 |
| Amx | 11 | 25 | 20 | Amp | 42 | 22 | 9 | 30 |
| Ap | 0 | 0 | 0 | Ap | .. | .. | .. | .. |
| Amc | 0 | 0 | 0 | Amc | .. | .. | .. | .. |
| C | 13 | 13 | 16 | C | 11 | 1 | 1 | 17 |
| Ft | 7 | 8 | 4 | Fl | 2 | .. | .. | .. |
| Na | 1 | 0 | 1 | Na | 1 | .. | 1 | .. |
| Ne | 2 | 7 | 6 | Ne | 24 | 13 | 1 | .. |
| Ot | 49 | 57 | 69 | Tet | 70 | 88 | 59 | 90 |
| S | 66 | 71 | 84 | S | 85 | 38 | 35 | 83 |
| Smx | 68 | 45 | 48 | Sul | 72 | 27 | 16 | 83 |
| Tmp | 5 | 16 | 13 | Tmp | .. | .. | .. | .. |

*Amc= amoxycillin+clavulanic acid, C= chloramphenicol, Na= nalidixic acid, S=streptomycin, Amp= ampicillin, Fl= furazolidone, Tet= tetracycline, Sul= sulphonamide, ref= references, ..= not tested, n= number of strains tested, see also legend Table 1.*

*Table 3: Comparison of the most frequently observed resistance patterns (%) of faecal Escherichia coli isolated from different groups of pig described in present thesis.*

| chapter | II (n= 387) | | IV (n=285) | | VII (n= 248) | |
|---|---|---|---|---|---|---|
| | pattern | % | pattern | % | pattern | % |
| | OtSSmx | 20 | OtS | 15 | OtS | 21 |
| | Smx | 12 | OtSSmx | 10 | OtSSmx | 14 |
| | SSmx | 11 | SSmx | 7 | S | 12 |
| | OtS | 8 | S | 5 | SSmx | 5 |
| | AmxOtSSmx | 3 | AmxOtSSmxTmp | 3 | AmxOtSSmxTmp | 3 |

*legend see Table 1.*

resistance to oxytetracycline and/or sulphamethoxazole, it is very likely that the usage of a tetracycline or sulphonamide might select for resistance to streptomycin (6,14,16).

The small variation in resistance to any of the antibiotics tested holds not only true for the prevalence and degree of resistance in the faecal samples, but also the antibiotic resistance of the individual *E. coli* isolates (the $MIC_{50}$ and $MIC_{90}$) of the group of pigs living at different farms and of the floor droppings collected from trucks were of the same order of magnitude (chapter VII).

In general it was shown that *E. coli* isolated from the intestinal flora of pigs were not only resistant to antibiotics commonly used for treatment of bacterial infections in humans, but even often a high degree of resistance could be observed against those

*Table 4: Comparison of the prevalence of antibiotic resistance (%) described for pigs in present thesis and by other investigators*

| | **Present thesis** | | | **Other studies** | | | |
|---|---|---|---|---|---|---|---|
| chapter | II | IV | VII | ref | 1 | 12 | 15 |
| n= | 407 | 291 | 286 | n= | 53 | 47 | 100 |
| | X (SD) | | | | | | |
| Amx | 81 (17) | 98 | 84 | Amp | 84 | 89 | 50 |
| Ft | 8 (13) | 17 | 4 | Fl | 6 | .. | 15 |
| Ne | 51 (25) | 92 | 73 | Ne | 47 | .. | 1 |
| Ot | 94 (10) | 100 | 89 | Tet | 94 | 98 | 100 |
| Smx | 94 (11) | 100 | 89 | Sul | 96 | .. | 89 |
| Tmp | 88 (18) | 99 | 77 | Tmp | .. | .. | 36 |

*See legend Tables 1 and 2.*

antibiotics: amoxycillin, tetracyclines, sulphonamides and trimethoprim.

Studies in other countries have also shown that the intestinal flora of healthy pigs is a reservoir of resistant bacteria (Tables 2 and 4). Differences in prevalences of resistance between these studies could be observed, but they might be explained by differences in sample selection, sampling procedures, laboratory methods and breakpoints used to differentiate between susceptible and resistant, but might also have been caused by differences in regional and local factors. These differences might be due to factors such as differences in prevalence of diseases, antibiotic usage, husbandry methods, hygienic measures, and/or disease control procedures. High resistance percentages to tetracyclines, sulphonamides and streptomycin, however, was commonly found in all these studies (Table 2). Hence, the faecal flora of healthy pigs represents a large reservoir of resistant bacteria. If these resistant bacteria are able to colonize the human gastro-intestinal tract or transfer their resistance genes to the human microflora, they might constitute a possible source of resistance for the human gastro-intestinal flora and consequently for bacteria (potentially) pathogenic for humans.

**Antibiotic resistance in healthy humans**

The prevalences and high degrees of antibiotic resistance in the stool samples of both abattoir workers and (sub)urban residents were of the same order of magnitude. Significantly higher values were found for the pig farmers (chapter III), although these were still lower than those noticed in the faecal samples of pigs (chapter IV).

A recognised important risk factor for the development of resistance in a population is the amount of antibiotic used in that population. Pig farmers are not only exposed to antibiotics directly by using these agents for treatment of personal infections, but also indirectly during treatment of pigs or handling medicated pig feed. Moreover, people may acquire resistant bacteria in their endogenous flora from their environment i.e. via contact with animals or animal products, but also from other sources such as vegetables. Pig farmers have daily and extensive contact with pigs and pig waste. Abattoir workers come regularly in contact with faecal bacteria during pig slaughtering or handling contaminated pig carcasses and meat. The difference between pig farmers and abattoir workers lies in the exposure to veterinary antibiotics (either directly or indirectly) by farmers as well as the intensity and time of contact with pigs and pig faeces.

Several other studies have reported higher prevalences of resistant bacteria in both

farmers and animal handlers than in people without, or with less intensive, animal contacts (8,9,12,14,18). These investigators suggested that contact with animals which had been treated with antibiotics was the cause of these higher prevalences. Only a few studies analyzed the recent usage of antibiotics for treatment of personal infections by the participants as one of the risk factors for antibiotic resistance (8,12). Recent antibiotic use for treatment of personal infections in this study was higher both in pig farmers and abattoir workers than in the (sub)urban residents (5% and 8% versus 0%, respectively). Abattoir workers were mostly treated locally for infections of minor skin wounds (dr. M.L.T. Quik v. Millingen, personal communication). The antibiotic use mentioned by pig farmers might be for treatment of chronic non-specific respiratory afflictions (CARA) which can be considered an occupational disease (13). This disease tends to cause regular acute exacerbations requiring antibiotic treatments. The regular use of antibiotics to treat these respiratory infections might have been a cause of the high prevalences of resistance in the faecal flora of the pig farmers. Attendant, contact and ingestion of medicated pig feed cannot be excluded as a risk factor for the presence of antibiotic resistance in the faecal flora of pig farmers. However, as described in chapter IV, ingestion of medicated pig feed seemed to be very unlikely to influence antibiotic resistance in pig farmers, as the theoretically daily ingestion of antibiotics was extremely low.

No significant differences were observed between abattoir workers who had intensive contact and those with less intensive contact, suggesting that minor differences in contact with pigs are not important. However, as pig farming is mainly a family business these people will usually have had contact with pigs and pig faeces for their entire lives, whereas the exposure to pig faecal flora can be expected to be much less for abattoir workers who work a regular 38 hrs week.

Consequently the higher prevalence of antibiotic resistance in pig farmers compared to the other human groups is multifactorial. Regular and intensive contact with pigs and pig faeces as well as long-term personal antibiotic use by pig farmers are possible factors of influence. As the faecal pig samples collected at different farms showed significantly higher prevalences of resistance than those of the pig farmer, the possibility of a flow of resistance genes from pigs to farmers might be suggested.

**Transfer of antibiotic resistance**

Further analysis, however, showed that in only 4% of the faecal pig and pig farmer samples, collected at the same farm, was the same resistance pattern present dominantly.

The most frequently resistance patterns encountered in pig farmer isolates were different from those most commonly found in the isolates from pigs. Pig farmer isolates mainly showed resistance to amoxycillin, sulphamethoxazole and streptomycin-sulphamethoxazole and pig isolates to oxytetracycline-streptomycin and oxytetracycline-streptomycin-sulphamethoxazole. Moreover plasmid transferability studies and plasmid DNA-finger printing did not show regular similarities between both groups, not even between paired pig and pig farmer strains. The plasmid profiles of plasmids isolated from pig farmer strains were quite different from those isolated from pig strains.

Differences in transferability *in vitro* between pig and pig farmer isolates were observed. Most pig strains could readily transfer resistance, whereas less than 50% of the pig farmer strains could transfer their resistance plasmids. *In vivo* transfer of resistance to a suitable acceptor was detected with a human as well as a pig donor strain. The data suggested that rats associated with a human intestinal flora permitted better transfer of resistance than in rat or pig flora associated rats.

In all the above data i.e. phenotypically only 4% of the isolates from the paired samples had the same resistance pattern, so that the genotypical differences in plasmid transferability *in vitro* and *in vivo* and the lack of similarity of plasmid DNA-fingerprints between the two groups strongly suggest that the faecal flora of pig farmers and pigs form more or less two separated pools of resistance genes with only limited exchange of resistance plasmids. O'Brien *et al.* (11) also showed that resistance plasmids isolated from human and animal (chicken) strains were highly diverse. In contrast, other investigators (4,17) found similarities among plasmids in animal and human isolates.

In a recent study in Sweden a dhfrIX-resistance gene mediating resistance to trimethoprim, widely spread among porcine isolates of *E. coli*, was detected only in one out of 434 trimethoprim resistant human *E. coli* strains. The authors concluded that transfer of resistance from pigs to humans is feasible (5).

**Antibiotic resistance surveillance in pigs**

It should be a goal in veterinary medicine to try to lower the carriage of antibiotic-resistant strains in animals. The data presented in this thesis did not show a major public health problem, but to safeguard the efficacy of antibiotics for treatment and prevention of bacterial diseases in animals now and for the future it is important to keep the carriage of antibiotic-resistant strains low (2,10). Taking their responsibility to achieve this, the

veterinary profession in The Netherlands has developed a veterinary antibiotic policy to prevent the emergence and dissemination of resistance as much as feasible, but without hampering optimal treatment of sick animals (2). The backbone of this veterinary antibiotic policy is one national antibiotic formulary: a limited list of preferred antibiotics, based on scientific data and practical experience. Essential, for the advice given in this formulary and in future updates, is regular information about trends in resistance development. Also for evaluation of the impact of the policy, regular surveillance of resistance in animals is necessary. Careful record keeping of all pathogens isolated from animals, complete with antibiotic susceptibilities, is important. However, as in veterinary medicine culturing and susceptibility testing is done mainly in case of treatment failure or from autopsy material, use of these data alone can lead to an exaggerated view of the prevalence of resistance in an animal population. It is therefore important to monitor regularly the prevalence and high degree of antibiotic resistance in the intestinal *E. coli* population in different groups of healthy animals to follow the effect of antibiotic usage in that population. A regular analysis of prevalence and high degree of resistance of the faecal flora and of isolated pathogenic bacteria by animal species, age group and organ system involved, should be made for different regions as well as nationally. This gives early warning of the emergence of resistance and/or cross-infection and clustering of resistant strains which may need special attention. A reliable, feasible and affordable method to obtain representative samples is therefore desirable.

In chapter VII prevalence and high degree of resistance as well as antibiotic susceptibility of *E. coli* isolated from faecal pig samples from individual farms were compared with the results obtained using floor droppings collected from trucks transporting fattening pigs from the same area as the farms to the slaughterhouse. Floor droppings yielded similar results and therefore collecting faeces samples at slaughterhouses seems to be an acceptable and much cheaper alternative than faeces collection on individual farms.

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## GENERAL CONCLUSIONS AND RECOMMENDATIONS

The results of this thesis suggest that the faecal flora of pigs and humans are different entities and constitute separate pools of resistance genes.

Although the intestinal *E. coli* flora of pig farmers showed higher prevalence of resistance percentages than those of (sub)urban residents and abattoir workers the data in the present thesis do not support the hypothesis that this difference is due to pig contact or involuntary intake of medicated pig feed. Another important finding is the observation that similar phenotypic antibiotic resistance patterns in the three populations studied were genotypically different.

Therefore, the question in the title of this thesis "Is there a relation between the antibiotic resistance in humans and pigs" can be answered. In our study this relation seemed to be negligible or even absent.

Although, in this thesis, pigs do not seem to be an important source of resistance for humans, it should be noted that the prevalence of resistance for the most commonly used antibiotics in pig medicine were extremely high, almost 100%. Just as in human medicine, it should be a challenge for the veterinary profession to try to lower the prevalence of resistance in the intestinal flora of domestic animals for several reasons. First to safeguard the efficacy of veterinary antibiotic therapy now and for the future and second to prevent possible public health implications. The Dutch veterinary profession has taken this responsibility seriously and a veterinary antibiotic policy has recently been published. To be able to evaluate the impact of this veterinary antibiotic policy active surveillance of antibiotic resistance in animal populations, such as pigs, has to be performed regionally and nationally. This surveillance is necessary to make scientifically based recommendations for regular updates of the antibiotic guidelines in the veterinary formulary and to keep an eye on emerging public health risks. National monitoring stations have to be established for active surveillance of antibiotic resistance in different groups of healthy animals. Since in the present study results obtained from faecal pig samples collected from trucks were of the same order of magnitude as those collected at individual farms, faecal samples collected at slaughterhouses can be used for the proposed surveillance of resistance in pigs. This surveillance will give early warning of the emergence of resistance and/or cross-infection and clustering of resistant strains in a certain area, which needs special attention. In addition, resistance prevalence can be directly related to the amounts of antibiotics used in a certain group of animals, as veterinary usage of antibiotics has to be recorded by the responsible veterinary surgeon and farmer.

Knowledge of the prevalence and degree of resistance in the bacterial flora of healthy animals will provide veterinary surgeons with essential data for their choice of empirical therapy and could alert them to optimize their use of antibiotics.

## SUMMARY

The aims of this thesis were to detect if, and to what extent, antibiotic resistance can be transferred from pigs to humans and whether contact with pigs, pig products or medicated pig feed constituted a possible public health risk. Therefore, the influence of direct contact with pigs or pig products as well as recent antibiotic use in human and pig medicine were investigated.

In the *introduction*, the literature is reviewed. The discovery of antibiotics had a major impact on the rate of survival from infections. However, the use of those agents resulted in antibiotic resistant bacterial populations in man and animals. Antibiotic resistance is common in bacteria, pathogenic as well as non-pathogenic, and is usually determined by plasmids. The prevalence of such plasmids and the range of antibiotics to which they confer resistance have increased greatly in the past 60 years. Resistance is common in the intestinal flora of healthy humans and animals. This flora acts as a reservoir of resistance genes which can be spread from one to the other population. The prevalence and dissemination of antibiotic resistance is a result of several factors which can be of direct or indirect influence. High risk groups are people living under poor hygienic conditions, living crowded together or using antibiotics regularly. People in close contact with food animals seem also to have a higher prevalence of resistance and therefore an attendant risk of spread of resistance from animals to man is expected. The occurrence of resistance in animals is directly related to the veterinary use of antibiotics, and this may indirectly influence resistance in humans. However, the literature is not clear about the extent of a possible public health risk of antibiotic use in veterinary medicine.

In *chapter II* the prevalence and high degree of antibiotic resistance of faecal *Enterobacteriaceae* and the susceptibility of *Escherichia coli* isolates from fattening pigs at one farm was studied in time. This study showed that healthy pigs represented a reservoir of resistant *Enterobacteriaceae* (*E. coli*). Antibiotic resistance was relatively constant in time for the pig population of this farm. Despite the absence of recent mass-medication during the stay at this farm, the prevalence of resistance to the most commonly used antibiotics in veterinary medicine was high. Susceptibility testing showed the highest resistance percentages for oxytetracycline, streptomycin and sulphamethoxazole, about half of the isolates showed patterns with resistance to sulphamethoxazole or a combinations of two or all of these antibiotics.

In *chapter III* the prevalence and high degree of antibiotic resistance of faecal *E. coli* isolated from pig farmers and abattoir workers were compared to a control group of

(sub)urban residents. The pig farmers showed the highest percentages and the (sub)urban residents the lowest. Except for neomycin, no significant differences in high degree of resistance were observed. Remarkably, 5% of the pig farmers and 8% of the abattoir workers mentioned recent antibiotic use, whereas none of the (sub)urban residents had taken antibiotics recently. This might indicate that people in contact with pigs or pig carcasses have a greater risk of bacterial infections. As abattoir workers with a different grade of pig contact showed no significantly different prevalence of resistance, contact with pigs or carcasses seemed to be of minor influence on antibiotic resistance in these persons. We thought originally that direct contact with antibiotics in medicated pig feed, i.e. mass-medication, influenced the prevalence of antibiotic resistance in pig farmers.

In *chapter IV* the prevalence and high degree of resistance of faecal *E. coli* isolated from pig farmers (as discussed also in chapter III) and their pigs as well as the susceptibility(patterns) are compared. For most antibiotics, pig *E. coli* isolates showed significantly higher prevalence of resistance percentages than the pig farmer strains. The pig isolates were mainly resistant to oxytetracycline-streptomycin/sulphamethoxazole, whereas the resistance patterns amoxycillin, sulphamethoxazole and streptomycin-sulphamethoxazole were observed most frequently for farmer strains. Only 4% of the faecal *E. coli* isolated from pig and pig farmer living at the same farm showed the same resistance pattern. The high use of antibiotics in pig medicine in general and the intensive faecal-oral contact between pigs might explain the higher resistance percentages observed in pigs in this study. The results suggest that the resistance of the faecal flora of pig farmers and their pigs is different.

*Chapter V* describes the results of *in vitro* transfer of antibiotic resistance plasmids from *E. coli* strains isolated from pigs and pig farmers living at the same farm, one group of isolates showed the same and the other group different resistance patterns. Besides similar transfer frequencies for pig farmer and pig strains, only minimal similarities could be observed with respect to biotypes, whole plasmid pattern and plasmid profile after restriction analysis. No conclusive evidence for the presence of a common pool of resistance plasmids among pig farmers and their pigs could be found.

*Chapter VI* describes the results of *in vivo* transfer of resistance plasmids present in porcine and human donor strains inoculated in germ-free rats associated with different *Enterobacteriaceae*-free intestinal floras. This study showed that *in vivo* transfer of resistance plasmids was possible in those rats and that the pig *E. coli* donor showed better transfer activity than the human donor. The origin of the intestinal flora seemed to influence the frequency of transfer. The human intestinal flora permitted better transfer of resistance: the highest number of transconjugants could be isolated from rats associated

with this flora. As expected, rat flora-associated rats seemed to inhibit transfer more efficiently, probably because of providing better colonization resistance.

In *chapter VII* the prevalence and high degree of resistance as well as the susceptibilities of *E. coli* isolates from pigs living at different farms are compared with the results of faecal floor droppings collected from trucks transporting fattening pigs. Both groups of isolates showed similar values, suggesting that floor droppings are useful for antibiotic surveillance in a pig population. A difference was observed in number of samples with overgrowth of *Bacillus* spp., probably due to floor-contamination with soil bacteria. To prevent this problem it might be advisable to collect faecal samples at the evisceration-line in the slaughterhouse instead of using floor droppings from trucks.

In *chapter VIII* the results, as presented in the chapters II to VII, are summarised and discussed and recommendations for the future are made.

This study showed that both pig farmers and pigs acted as reservoirs of resistance genes. They had to be seen as different entities and constituted separate pools of resistance genes, although *in vivo* exchange of resistance plasmids between porcine and human strains was possible. Antibiotic resistance is multifactorial of origin. This study showed that minimal influence on resistance could be expected by contact with pigs, pig products and waste and after intake of medicated pig feed. Usage of antibiotics for personal treatment could be a cause of the high resistance observed in pigfarmers.

# SAMENVATTING

De vraagstellingen van dit proefschrift waren of, en in welke mate, antibiotica-resistentie kan worden overgedragen van varkens naar mensen en of contact met varkens, varkensproducten en gemedicineerd varkensvoer een mogelijk gevaar vormen voor de algemene gezondheid. Daarom werd zowel de invloed van direct contact tussen mensen en varkens of varkensprodukten als het recente antibioticum gebruik in de humane- en diergeneeskunde onderzocht.

In de *introductie* wordt een overzicht van de literatuur gegeven. Het gebruik van antibiotica was hoofdzakelijk bedoeld voor het overwinnen van infectie-ziekten. Eén van de consequenties van de toepassing van deze stoffen was echter de vorming van bacteriënpopulaties in mens en dier die resistent waren tegen deze antibiotica. Antibiotica-resistentie is algemeen voorkomend in zowel pathogene als niet pathogene bacteriën en wordt hoofdzakelijk bepaald door plasmiden. Gedurende de afgelopen 60 jaren was er een enorme toename in het voorkomen van resistentie-plasmiden en het aantal antibiotica waartegen resistentie bestaat. Resistentie is algemeen voorkomend in de darmflora van gezonde personen en dieren. Deze flora is een reservoir van resistentie-genen die van de ene naar de andere populatie kunnen worden overgedragen. Het voorkomen en de verspreiding van antibiotica-resistentie wordt bepaald door meerdere factoren die direct of indirect van invloed kunnen zijn.

Mensen die leven onder slechte hygiënische omstandigheden, in dichtbevolkte gebieden of die regelmatig antibiotica gebruiken vormen hoge risicogroepen. Personen die contact hebben met consumptiedieren lijken ook hogere resistentiewaarden te hebben, waardoor een bijkomend risico van resistentie verspreiding van dier naar mens wordt verondersteld. Het voorkomen van resistentie in dieren is direct gerelateerd aan het diergeneeskundig antibioticum gebruik en kan eventueel indirect van invloed zijn op het voorkomen van resistentie bij de mens. De literatuur geeft echter geen opheldering over de aanwezigheid van een mogelijk gezondheidsrisico ten gevolge van diergeneeskundig antibiotica gebruik.

In *hoofdstuk II* worden de prevalentie en de hoge mate van resistentie van faecale *Enterobacteriaceae* en de gevoeligheid van *Escherichia coli* geïsoleerd van mestvarkens op één bedrijf, beschreven in functie van de tijd. Deze studie toont aan dat gezonde varkens een reservoir van resistente *Enterobacteriaceae* (*E. coli*) vormen. De antibiotica-resistentie was voor de varkenspopulatie op dit bedrijf relatief constant gedurende de tijd. Ondanks de afwezigheid van groepsmedicatie gedurende het verblijf op dit bedrijf was de prevalentie van resistentie voor de meest frequent gebruikte antibiotica in de diergeneeskunde

hoog. De hoogste resistentie percentages werden waargenomen voor oxytetracycline, streptomycine en sulfamethoxazole, de resistentie patronen van ongeveer de helft van de isolaten vertoonden een combinatie van twee of drie van deze antibiotica of alleen resistentie tegen sulfamethoxazole.

In *hoofdstuk III* worden de prevalentie en de hoge mate van resistentie van faecale *E. coli*, geïsoleerd van varkenshouders en slachthuispersoneel, vergeleken met een controlegroep bestaande uit isolaten van stedelingen. De varkenshouders vertoonden de hoogste percentages en de stedelingen de laagste. Met uitzondering van neomycine werden er geen significante verschillen in de hoge mate van resistentie waargenomen. Opvallend was dat 5% van de varkenshouders en 8% van het slachthuispersoneel recent antibiotica hadden gebruikt, terwijl geen van de stedelingen recent antibioticum gebruik vermelden. Dit kan erop duiden dat mensen in contact met varkens of varkenskarkassen een groter risico op bacteriële infecties hebben. Aangezien slachthuispersoneel met verschillende mate van varkenscontact geen significante verschillen in prevalenties vertoonden lijkt het dat contact met varkens of varkenskarkassen van beperkte invloed is op antibiotica-resistentie in deze groep. Aanvankelijk dachten we dat direct contact met antibiotica in gemedicineerd varkensvoer (groepsmedicatie) van invloed was op de prevalentie van antibiotica-resistentie in varkenshouders.

In *hoofdstuk IV* worden, naast de prevalentie en de hoge mate van resistentie, de gevoeligheidspatronen van faecale *E. coli* geïsoleerd van varkenshouders (zoals besproken in hoofdstuk III) en hun varkens vergeleken. Varkens *E. coli* isolaten vertoonden voor de meeste antibiotica significant hogere prevalentie percentages dan de stammen van de varkenshouders. De varkens isolaten waren hoofdzakelijk resistent voor oxytetracycline-streptomycine/sulfamethoxazole, terwijl voor de varkenshouders stammen hoofdzakelijk de resistentie patronen amoxycilline, sulfamethoxazole en streptomycine-sulfamethoxazole werden waargenomen. Bijkomend vertoonden enkel 4% van de faecale *E. coli* isolaten van varkens en varkenshouders afkomstig van hetzelfde bedrijf dezelfde resistentie patronen. De hogere resistentie percentages waargenomen voor de varkens, zouden verklaard kunnen worden door het veelvuldig gebruik van antibiotica in de varkensgeneeskunde en het intensief faecaal-oraal contact tussen varkens. De resultaten suggereren een verschil in resistentie van de faecale flora van varkenshouders en varkens.

*Hoofdstuk V* beschrijft de resultaten van *in vitro* overdracht van resistentie-plasmiden van *E. coli* stammen geïsoleerd van varkens en varkenshouders afkomstig van hetzelfde bedrijf, een groep isolaten vertoonde dezelfde en de andere groep verschillende resistentie patronen. Behalve vergelijkbare overdrachtsfrequenties voor de varkenshouders- en varkensstammen, konden enkel geringe overeenkomsten in biotypen, volledig plasmidpa-

troon en plasmidprofiel na restrictie-analyse worden waargenomen. Deze studie levert geen duidelijk bewijs voor de aanwezigheid van één gemeenschappelijke poel van resistentieplasmiden voor varkenshouders en varkens.

*Hoofdstuk VI* beschrijft de resultaten van de *in vivo* overdracht van resistentie-plasmiden van een varkens en een humane donorstam geïnoculeerd in kiemvrije ratten welke geassocieerd waren met verschillende *Enterobacteriaceae*-vrije darmflora's. De studie liet zien dat *in vivo* overdracht van resistentie-plasmiden mogelijk was in deze ratten. De varkens *E. coli* donor vertoonde een betere overdracht dan de humane donor. De soort darmflora leek van invloed te zijn op de overdrachtsfrequentie. De humane darmflora liet een betere resistentie-overdracht toe: het hoogste aantal transconjuganten kon worden geïsoleerd van ratten geassocieerd met deze flora. Zoals verwacht leek het dat in ratten geassocieerd met rattenflora de overdracht effectiever geremd werd, waarschijnlijk ten gevolge van de aanwezigheid van een betere kolonisatie-resistentie.

In *hoofdstuk VII* werden de prevalentie en de hoge mate van resistentie evenals de gevoeligheid van *E. coli* isolaten van varkens afkomstig van verschillende bedrijven vergeleken met mestmonsters verzameld van de bodem van vrachtwagens die de varkens naar het slachthuis transporteerden. De overeenkomstige waarden waargenomen voor beide groepen suggereren dat vrachtwagenmonsters bruikbaar zijn voor de observatie (surveillance) van antibiotica-resistentie in een varkenspopulatie. Wel was er een verschil in aantal monsters die *Bacillus* spp. overgroei vertoonden, waarschijnlijk toe te schrijven aan contaminatie met bodembacteriën. Om dit probleem te voorkomen lijkt het raadzaam om, in plaats van bodemmonsters, in het slachthuis faeces te verzamelen aan de eviscera-tielijn.

In *hoofdstuk VIII* worden de resultaten, beschreven in de hoofdstukken II tot en met VII, besproken en bediscussieerd en worden aanbevelingen voor de toekomst gedaan.

Deze studie toonde aan dat varkenshouders en varkens reservoirs van resistentie-genen vormen. Zij moeten beschouwd worden als verschillende entiteiten die afzonderlijke poelen van resistentie-genen vormen, alhoewel *in vivo* uitwisseling van resistentie-plasmiden mogelijk is. De oorsprong van antibiotica-resistentie is multifactorieel. Factoren zoals contact met varkens, varkensprodukten, faeces en opname van gemedici-neerd varkensvoer lijken van geringe invloed te zijn op het voorkomen van antibiotica-resistentie in de onderzochte personen. Het gebruik van antibiotica voor eigen therapie is een mogelijke oorzaak van de hogere resistentie waarden waargenomen voor de varkens-houders.

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# CURRICULUM VITAE

Ruth Nijsten werd geboren op 19 februari 1963 te Bunde. In 1982 behaalde zij het Atheneum-B diploma aan scholengemeenschap Stella Maris te Meerssen.
Na in Nederland te zijn uitgeloot voor de studie diergeneeskunde begon zij in oktober 1982 aan deze studie aan het Rijks Universitair Centrum Antwerpen te België en behaalde het dierenartsexamen in 1988. Het eerste anderhalve jaar na het afstuderen nam zij waar in diverse praktijken voor gezelschapsdieren. Vervolgens heeft zij gedurende een jaar gewerkt als praktizerend dierenarts in een praktijk voor gezelschapsdieren. Na twee maanden werkzaam te zijn geweest als keuringsdierenarts bij de Rijksdienst voor keuring van Vee en Vlees trad zij 1 mei 1991 in dienst als toegevoegd onderzoeker bij de vakgroep Medische Microbiologie van de Rijksuniversiteit Limburg. In de afgelopen drie en een half jaar werd onder begeleiding van dr. E. Stobberingh en drs. A. van den Bogaard door het Praeventiefonds gesubsidieerd onderzoek verricht. De bevindingen zijn beschreven in dit proefschrift.